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13. ABSTRACT (Maximum 200 Words)

The growth of mammary tumors reflects the partial or total compromise of tumor specific immune surveillance in the tumor bearing host. By following the function and fate of T cells that recognize a defined antigen, it is possible to define the mechanisms involved in immune evasion by the tumor, and to open possibilities for targeted therapy. In the previous annual report we described an animal model of autochtonous mammary cancer. It was also reported that transgenic expression of an ectopic antigen (influenza hemagglutinin: HA) led to the generation of HA specific regulatory T cells, and that this was directly linked to the spurious intrathymic expression of this antigen. Over the course of the past year, we have made progress in generating a novel knock-in mouse line, that when crossed to tissue specific Cre mice allows for tight tissue specific expression of HA. We have also demonstrated that in the presence of physiological levels of tumor specific regulatory T cells anti-tumor cytolytic T cell responses are severely suppressed, while homing and proliferation of the T cells are apparently unaffected. Furthermore, we have shown that lymphonic expansion of CD8 T cells activates them and renders them resistant to regulation. These studies elucidate tolerance mechanisms that may be safeguarding immunogenic tumors against potentially lethal host T cell responses, and direct us to therapeutic strategies.

14. SUBJECT TERMS

Whey Acidic Protein, Cre/loxP, β-catenin, mammary-tumor, tumor-antigen, T cell receptor transgenic mice, T cell/ bone marrow reconstitution, tolerance, immune reactivity, therapy.

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INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The growth of mammary tumors reflects the partial or total compromise of tumor specific immune surveillance in the tumor bearing host. Failure of antitumor T cell responses are likely to have both systemic and local causes. Spurious expression of tumor antigens in the thymus could lead to the generation of regulatory T cells, which in turn may suppress specific cytolytic T cells responses. At the site of the tumor, local tissue and tumor microenvironment could also influence tumor-T cell interactions and outcome. By following the function and fate of T cells that recognize a defined antigen, it is possible to define the mechanisms involved in immune evasion by the tumor, and to open possibilities for targeted therapy. In the previous annual report we described an animal model of autochtonous mammary cancer. It was also reported that transgenic expression of an ectopic antigen (influenza hemagglutinin: HA) led to the generation of HA specific regulatory T cells, and that this was directly linked to the spurious intrathymic expression of this antigen. Over the course of the past year, we have made progress in generating a novel knock-in mouse line, that when crossed to tissue specific Cre mice allows for tight tissue specific expression of HA. We have also investigated in detail the impact of the presence of physiological levels of tumor specific regulatory T cells on anti-tumor cytolytic T cell responses. To this aim we have used HA specific $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ regulatory T cells, together with transplantable tumors expressing the HA surrogate tumor antigen. These studies have enabled us to elucidate tolerance mechanisms that may be responsible for safeguarding immunogenic tumors against potentially lethal host T cell responses. We expect to extend these studies to autochtonous tumors in the coming year.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the **approved** Statement Of Work.

Task 1: To express the influenza HA as a neo-self antigen in the mammary alveolar cells *in vivo*.

Last years' report described our use of the Cre/loxP recombination system to construct a mouse with ubiquitous but inducible expression of influenza HA, namely the ($PGK^{loxP}lacZ^{loxP}HA$) mice. These were crossed to mice expressing an HA-specific transgenic T cell receptor on CD4 T cells (TCR6.5), and mice that express Cre in a mammary specific manner. The lymphoid phenotype of ($PGK^{loxP}lacZ^{loxP}HA$ x WAPCre x TCR6.5) compound mutant mice was dramatically different from that of the single TCR transgenic mice. Thymic cellularity was diminished by about 50%, and among thymic $CD4^+$ single-positives, TCR6.5+ cells were reduced from 38% to 16%, totaling in a four-fold reduction of 6.5+ CD4+ T-cells. In mesenteric lymph nodes and spleen the proportion of TCR-6.5 expressing cells among CD4 T cells was very small (2% versus 12% in controls). Partial depletion of HA specific T cells was also observed in the lymph nodes and spleen. This unexpected widespread loss of HA specific T cells prompted a re-screening of the double transgenic WAP-Cre x $PGK^{loxP}lacZ^{loxP}HA$ mice for tissue specific recombination and antigen expression. It was surprising to find germ line recombination in nearly all WAP-Cre x $PGK^{loxP}lacZ^{loxP}HA$ double transgenic mice. A most plausible explanation was differences in relative susceptibilities of different genetic loci to Cre/loxP mediated recombination. We had not observed germline recombination in crosses of Wap-Cre to the R26R mice that express lacZ from the ROSA26 locus, in a Cre dependent manner. Presumably, in our founder line the HA cassette must have inserted in a locus that is more prone to Cre mediated recombination than the ROSA-26 locus. To overcome the problem, we have generated novel knock-in mice, in which the HA cassette is inserted in the ROSA-26 locus.

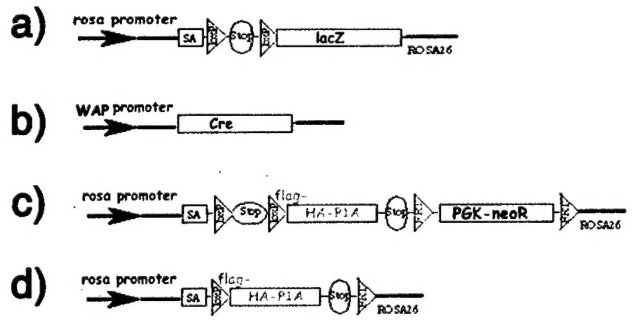

Figure 1. Schematic representation of the a) R26R, b) WAP-Cre, c) HA-P1A in the ROSA-26 locus, before Cre mediated recombination, and d) after recombination. In R26R, a transcription stop signal is flanked by loxP sequences, and can be excised by Cre mediated recombination. Cre mediated removal of the stop sequence allows expression of lacZ, driven by the ROSA promoter. Similar to the R26R, the HA-P1A cassette recombines in the presence of Cre to express HA-P1A. Rectangles represent loxP sites, and arrows indicate promoters.

The new mice express a fusion of HA with a mini P1A gene, to allow comparison of immune responses against the viral antigen (HA) with those against a true murine tumor antigen (P1A). This mini-P1A gene (consisting of codons 11-67 fused to the last P1A codon, the stop codon and polyadenylation signal sequence) was amplified by asymmetric PCR using P1A cDNA as template, and was fused to the 3' end of HA gene. A flag-tag peptide (DYKDDDDK) was fused to the amino terminal end of HA to facilitate detection of the antigens, Besides, a translation/transcription termination signal derived from the human growth hormone gene (hGH stop signal sequence) was cloned downstream of the flag-HA-P1A fusion for proper termination of gene expression.

To render its expression conditional on Cre activity, the pBKS stop cassette was flanked by loxP sequences, and then cloned upstream of the HA-P1A mini gene. The pBKS is a strong translation/transcription signal that prevents unwanted expression of downstream genes. It consists of transcriptional termination elements derived from the C-terminal sequences of the yeast *HIS3* gene, followed by an SV40 polyadenylation signal, as well as a synthetic oligonucleotide including a false translation initiation signal and a 5' splicing donor site providing additional safeguards. To allow for selection of cells harboring the above sequences a neomycin resistance mini gene was included under the control of the phosphoglucokinase promoter PGK-neo cassette. The PGK-neo min-gene was flanked by FRT sequences to allow its removal by flp recombinase, and was subcloned 3' to the hGH stop signal sequence. The entire cassette described above was flanked by homologous sequences from the ROSA26 gene, to facilitate targeted recombination and placing of gene expression under the control of the ROSA26 gene promoter. For this purpose, we have chosen a by now well known strategy: the cassette was targeted downstream of the first untranslated exon of the ROSA26 gene, such that proper splicing events in the resulting mRNA, fuses the exon with the sequences derived form our cassette. For this purpose, a splicing acceptor sequence (SA) was amplified by PCR using pSABgeo (provided by Dr. Soriano P.) as template and inserted upstream of 5' loxp site of pBKS stop cassette. This final cassette was subcloned into ROSA26 targeting vector (provided by Dr. Soriano P., and modified in the lab). In the final modified locus, the flag-HA-P1A is expressed upon Cre/loxP mediated recombination and excision of the pBKS stop sequence (Figure 1, c and d).

The exchange cassette was transfected into BALB/c derived embryonic stem cells (ES) (provided by Dr. Klaus Rajewsky), and homologous recombined ES clones were identified by combination of PCR and southern blot analysis (Figure 2). Three ES clones (D6FLP1, F9FLP2 and F9FLP5) were provided to transgenic facilities at Harvard for injection into C57BL/6 blastocystes. Two independent male chimeric mice, with 45% and 25% chimerisms were produced. These were backcrossed to BALB/c mice. From the cross of the 45% chimeric male mouse we have obtained 6 white pups, at least one of which is positive in a preliminary PCR screening for the presence of the HA cassette. Currently, we are screening these F1 generation pups by Southern-Blot analysis for the presence of the stop-HA cassette. Positive pups will be backcrossed to WAP-Cre /Thy1.1 mice and to WAP-Cre/thy1.1/ $Catnb^{+/lox(ex3)}$ mice, and these in turn will serve as recipients of HA specific TCR transgenic CD8 and CD4 T cells, from Thy1.2 donors. Interaction of antigen specific T cells and mammary/tumor epithelium will be followed, taking advantage of the Thy1.1 allele difference of the transferred T cells and host cells.

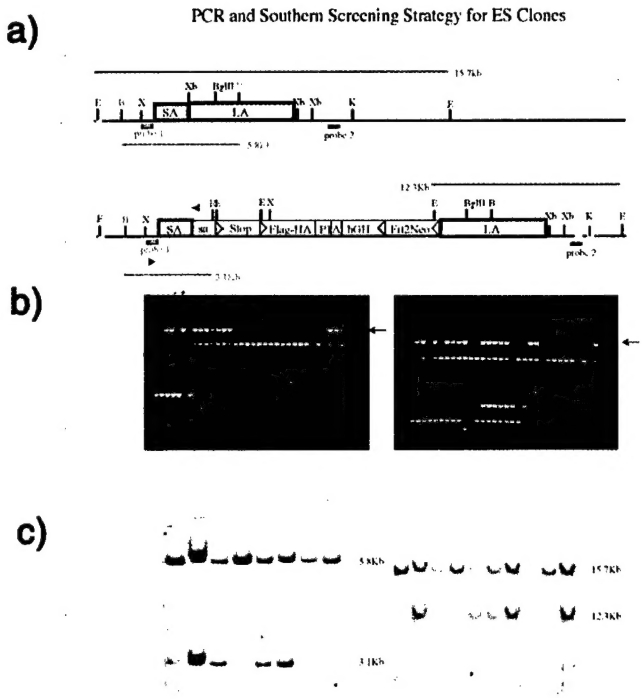

Figure 2. **Schematic representation of constructs, PCR and Southern Blot screening strategy of ES transfectants of stop-HA insertion cassette.** (a) Restriction map and southern blot screening strategy of wild type (upper scheme) and recombined allele (lower scheme) of the ROSA26 locus. By southern blot, probe1 (pink color) visualizes bands of 5.8Kb and 3.1Kb sizes, corresponding to the wild type Balb/c and recombined alleles respectively; probe2 (blue color) recognizes bands of 15.7Kb and a 12.3Kb sizes corresponding to the wild type and recombined alleles respectively. (b) PCR screening of ES clones transfected with the stop-HA insertion cassette; black arrows indicate the 1.2 Kb PCR product amplified from the homologously recombined cassette; primers used for the PCR recognize the endogenous ROSA sequence and newly introduced sequence, as shown schematically by red arrowheads in panel (a). (c) Southern Blot analysis of ES clones transfected with the stop-HA insertion cassette, using probe1 (left panel) or probe 2 (right panel).

The above approach is based on our preliminary observations indicating that the ROSA26 locus is faithful for tissue specific Cre mediated recombination. Mice expressing lacZ in a Cre dependent manner under the control of the ROSA26 promoter, the R26R mice, are already available and characterized (1). An alternative approach is to use these mice in combination with lacZ specific TCR transgenic mice. To this aim have obtained lacZ specific CD4 and CD8 TCR transgenic mice from the laboratory of Dr. Restifo (unpublished) at NIH. The TCR transgenics were characterized for specific recognition of lacZ epitopes, by adoptive transfer of CFSE labeled cells into recipient mice and vaccination of these mice with purified β-galactosidase protein in incomplete Freunds' adjuvant (IFA). The preliminary characterization of the mice indicates that both CD4 and CD8 TCR transgenic T cells readily recognize lacZ specific epitopes, and are activated and proliferate in the lymph nodes draining the vaccination site. The mice are therefore suitable for our study. To facilitate detection of the adoptively transferred T cells in cancer prone mice, the TCR transgenic mice have been backcrossed to the C57BL/6, Ly5.1, Rag1 background. This will allow us to use Ly5.1 specific antibody to follow the transferred T dells in C57BL/6 Ly5.2 recipient mice. Furthermore, the $Rag1^{-/-}$ background will facilitate purification of homogenously lacZ specific T cells for our studies.

Task 2: To predictably and reproducibly initiate mammary tumors expressing HA in mice.

Mammary specific stabilization of β-catenin and rapid initiation of mammary lesions in an inducible manner was reported by us in Proc Nat Acad Sci USA (2002) 99: 219-224.

Task 3: To investigate the immunological consequences of neo-self antigen/surrogate tumor antigen.

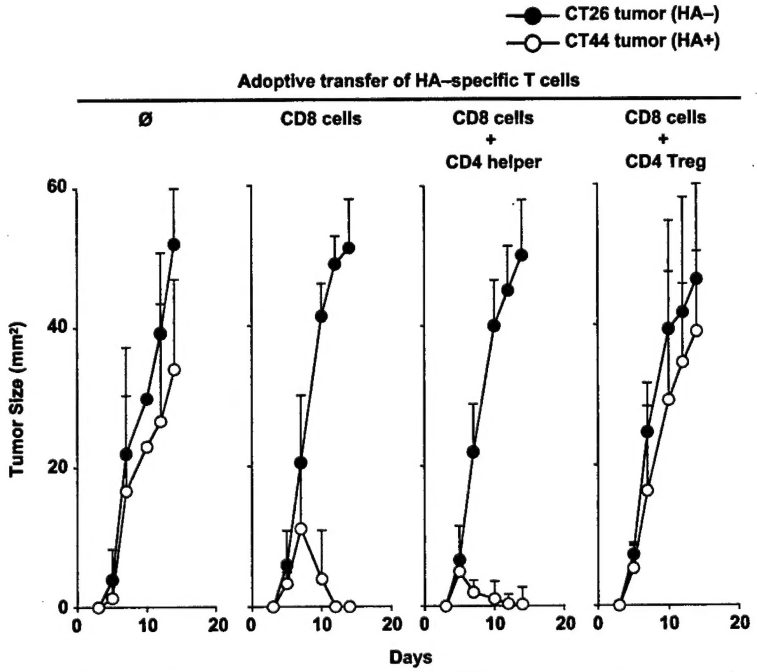

Figure 3 – HA–specific CD8 T cells specifically reject HA–expressing tumors in the absence of Treg. 10^5 T cells were adoptively transferred one day prior s.c. innoculation of 10^6 tumor cells (CT26 (HA–) tumor cells, right footpad, and CT44 (HA+) tumor cells, left footpad).

The exact mechanisms compromising specific immune responses in cancer patients are likely to differ from tissue to tissue and the type of cell affected. However, a common denominator in all cancers is the promiscuous expression of tissue and tumor specific genes in the thymus. Recent observations in transgenic mice suggest promiscuous expression in the thymus of particular tumor antigens (2). One consequence of this may

be the intrathymic generation of regulatory T cells that suppress immune responses against tissue/tumor specific antigens (3); for reviews see (4-6). Indeed, adoptive T cell transfer together with myeloablative treatment have provided promising results in the treatment of melanoma patients (7), supporting the view that depletion of regulatory cells prior to immunotherapy can be beneficial, in controlling cancer.

Our observations (see last years' report) indicate that spurious expression of tissue/tumor specific antigens in the thymus leads to the generation of antigen specific regulatory T cells. To reveal the significance of T cell regulation on the host anti-HA immune response, we performed adoptive transfer of HA specific regulatory T cells, to tumor bearing mice. The initial experiments aimed at using an experimental system that allowed investigation of the behavior of antigen–specific CD4 regulatory T cells (Treg) in an otherwise unperturbed immune environment. For this purpose, naïve/resting TCR Transgenic CD8 T cells, CD4 helper T cells and/or CD4 Treg, all directed against HA, were adoptively transferred into Balb/c mice, that were subsequently challenged with tumors expressing or not expressing HA (CT44 and CT26 tumor cell lines respectively(3)).

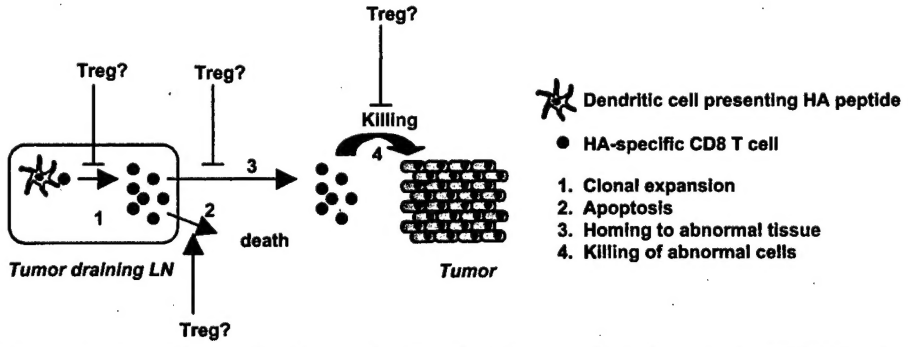

Figure 4 – Possible mechanisms of action for Treg-mediated control of CD8 T cell responses.

Inhibition of CD8 T cell immune responses by Treg may occur at different levels (Fig. 4), including: (A) control of CD8 T cell priming and proliferation, (B) acceleration of apoptosis, (C) inhibition of CD8 T cell homing to target sites, and (D) control of CD8 T cell anti-tumor effector functions.

Indeed, adoptively transferred naïve TCR transgenic CD8 T cells directed against the I-E_d–restricted $HA_{107-119}$ peptide (thereafter, HA–specific CD8 T cells(3)) rejected HA^+ (CT44), but not HA^- (CT26) tumors. Rejection of CT44 tumors occurred faster when HA–specific CD8 T cells were transferred together with naïve TCR transgenic CD4 T cells directed against the K_d–restricted $HA_{512-520}$ peptide (thereafter, HA–specific CD4 Helper T cells (3)). In marked contrast, however, when HA–specific CD8 T cells were transferred together with TCR Tg $CD4^+$ $CD25^+$ T cells from *pgk-HA* x *TCR-HA* mice (thereafter, HA–specific CD4 Treg (3)), CD8 T cell–mediated rejection of HA^+ tumors was abolished (Figure 5).

Because CD4 Treg have been shown to impair CD8 T cell proliferation *in vitro* (8), we first thought that a similar mechanism might contribute to control CD8 T cell-mediated immune responses *in vivo*. We adoptively transferred Thy1.1 Balb/c mice with

CFSE–labeled HA–specific Thy1.2 CD8 T cells in presence or absence of either HA–specific CD4 Helper or CD4 Treg. Mice were challenged with CT44 and CT26 simultaneously, and the tumor draining LNs were collected at different time points.

The percentage of transferred naïve HA–specific CD8 T cells represented <0.01% of the CD8 T cell pool at the time of tumor challenge (Fig. 6a), and increased up to 1–3% 6–8 days later. Proliferation of HA–specific CD8 T cells occurred in the CT44 tumor draining LN, but not in the CT26 tumor draining LN, where cells remained at very low frequencies and undivided (Fig. 6b). Surprisingly, however, the number of HA–specific CD8 T cells found in the course of the immune response in the CT44 tumor draining LN was not altered in the presence of CD4 Treg (Fig. 6a), excluding the possibility that Treg controlled CD8 T cell priming *in vivo*. When co-transferred with HA–specific CD8 T cells, HA–specific CD4 Treg (and CD4 Helper) also extensively proliferated in the CT44 tumor draining LN, but not in the CT26 tumor draining LN (data not shown), confirming that both class I and class II HA–restricted peptides were presented by professional antigen-presenting cells in the LN draining the HA^+ tumor.

Furthermore, detailed analysis of HA–specific CD8 T cells showed that HA–specific CD4 Treg did not impair their proliferation. Indeed, 98–99% of the transferred CD8 T

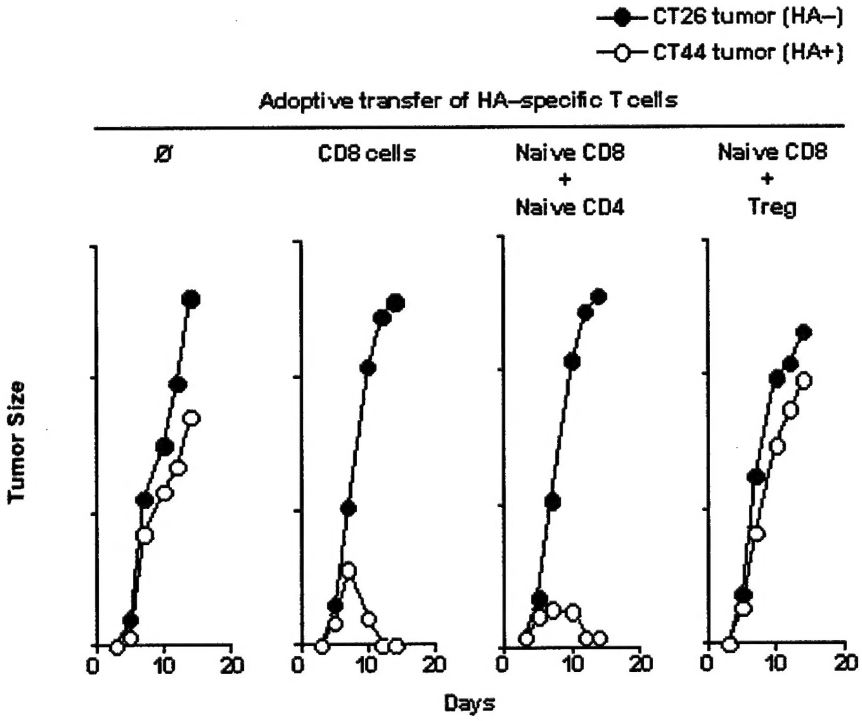

Figure 5– Tumor–specific CD8 T cells selectively reject HA–expressing tumors in the absence of tumor–specific Treg. T cells were adoptively transferred one day prior s.c. inoculation of tumor cells (CT26 (HA–) colon carcinoma, right footpad, and CT44 (HA+) colon carcinoma, left footpad). Tumor size was measured every second day. Results show mean and S.D. values of ≥ 5 mice.

cells had homogeneously and extensively divided 6 days after tumor challenge, even in the presence of Treg (Fig. 6 a–b). Similarly, HA–specific CD4 Treg did not accelerate apoptosis of HA–specific CD8 T cells, as similar numbers of transferred CD8 T cells were stained with Annexin V in the absence or presence of Treg (Fig. 5b). Similar observations were made 4 days and 8 days after tumor challenge (data not shown).

Although proliferation and apoptosis rates of HA–specific CD8 T cells were not altered by Treg *in vivo*, the CD25 expression by activated HA–specific CD8 T cells was markedly reduced in the presence of Treg. For instance, HA–specific CD8 T cells strongly upregulated CD25 expression at day 6 in the presence of HA–specific CD4 T Helper cells, whereas the latter cells remained $CD25^-$ (Fig. 6c). In marked contrast, HA–specific CD8 T cells only slightly upregulated CD25 expression in the presence of HA–specific CD4 Treg, whereas the latter cells remained $CD25^+$ (Fig. 6c). Hence, we

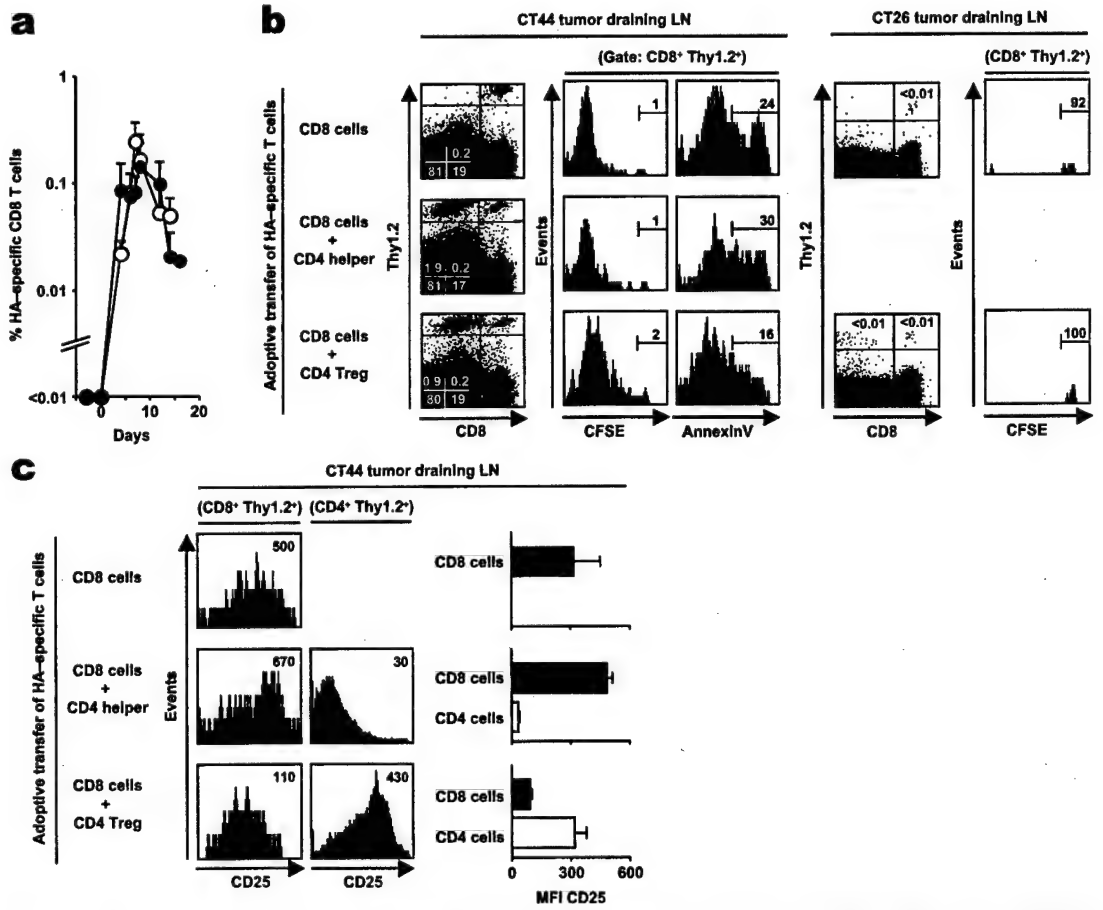

Figure 6. Tumor–specific Treg in tumor draining LNs neither impair proliferation nor accelerate apoptosis of tumor-specific CD8 T cells. (a) CFSE-labeled Thy1.2 HA–specific CD8 T cells were adoptively transferred into Thy1.1 recipients either alone (○) or together with Treg (●) one day before tumor inoculation as described in Figure 1. LNs draining the CT44 (HA+) and CT26 (HA–) tumors were collected at different time points and the transferred HA–specific CD8 T cells were quantified by flow cytometry. (b) At day 6, transferred CD8 T cells were assessed for proliferation (CFSE expression) and apoptosis (binding to Annexin V). Similar results were obtained at days 4 and 8 (data not shown). (c) At day 6, transferred CD4 and CD8 T cells were assessed for CD25 expression

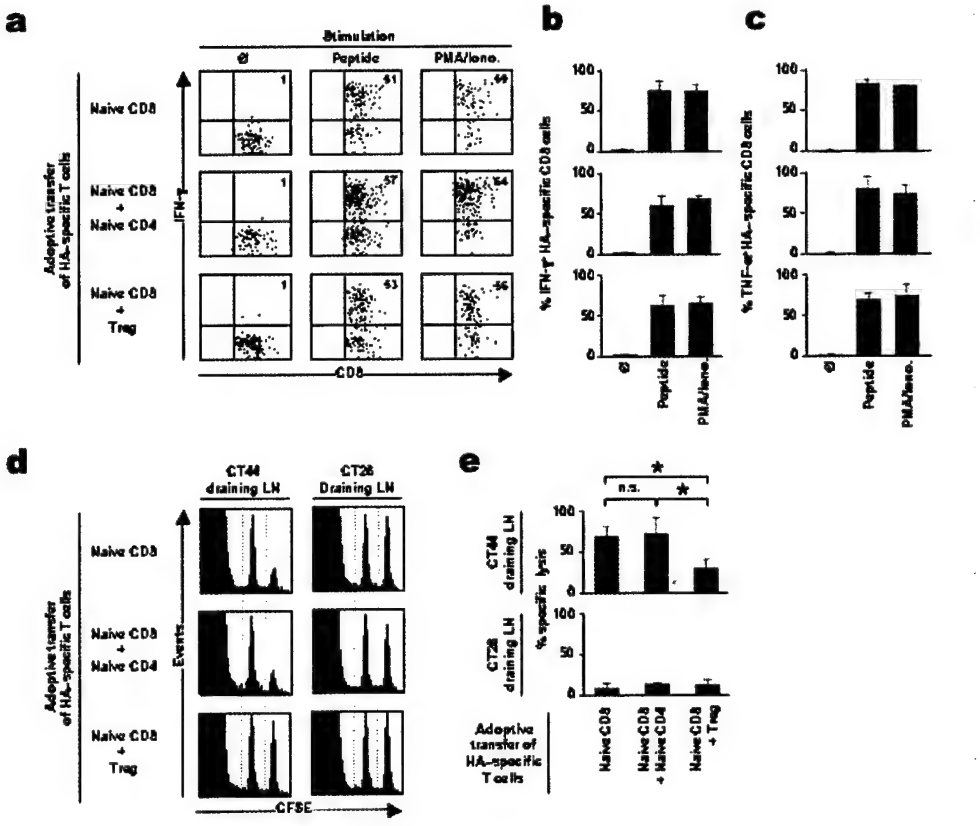

Figure 7 – Treg selectively control CD8 T-cell mediated cytotoxicity. Thy1.2 HA–specific T cells were adoptively transferred into Thy1.1 recipients one day before tumor inoculation as described in Figure 1. (a–c) Tumor draining LNs were collected at day 6 and were subjected to restimulation for 4 h with either (i) Ø, (ii) Class I and II–restricted HA peptides, or (iii) PMA and Ionomycin. HA-specific CD8 T cells were analyzed for production of IFN-gamma (a and b) and TNF-alpha (c). (d–e) At day 5, mice were injected with syngeneic splenocytes previously labeled with either (i) intermediate dose of CFSE and no peptide or (ii) high dose of CFSE and 1 μg/ml HA-peptide (the two populations were mixed at a 1:1 ratio before transfer). CT44 and CT26 tumor draining LNs were collected 16 hours later. Percentage specific lysis was calculated as follows: % specific lysis = 100 – ((%CFSE^{bright}/%CFSE^{low}) X 100). N.s., not significant, *, p<0.0001.

concluded that the differentiation status of HA–specific CD8 T cells might have been altered in the presence of Treg.

Because HA–specific Treg did not impair priming of naive CD8 T cells, we further investigated whether the functional status of the CD8 T cell progeny was influenced by Treg. To trigger an efficient immune response, the antigen-primed progeny includes effector CD8 T cells, which gain the ability to migrate to peripheral tissues and act by releasing inflammatory cytokines, which recruit macrophages and neutrophils and generate a local inflammatory response, and/or lytic molecules, which mediate target cell apoptosis(9). We show that the majority of HA–specific CD8 T cells in CT44 tumor draining LN produced IFN-γ (Fig. 7a–b) and TNF-α (Fig. 7c) upon 4 h restimulation with

peptide or PMA/Ionomcyin *ex vivo*. Furthermore, activated Treg in the CT44 tumor draining LN did not control secretion of the inflammatory cytokines by HA–specific CD8 T cells.

HA–specific CD8 T cells exhibited potent cytolytic activity *in vivo* in the absence of Treg, as demonstrated by antigen–specific killing of CFSE–labeled HA peptide–pulsed syngeneic splenocytes retrieved in CT44 tumor draining LNs (Fig. 6d–e). In contrast, however, the cytotolytic activity of HA–specific CD8 T cells was markedly impaired in the presence of Treg ($p<0.0001$, Fig. 4e). Killing of HA^+ targets was not observed (or only to a very low extend) in CT26 tumor draining LNs (Fig. 6d–e), in popliteal LNs of non-tumor challenged mice (data not shown), and in CT44 tumor draining LNs from CT44-bearing mice that did not receive HA–specific CD8 T cells (data not shown).

The observations may have revealed a novel mechanism for Treg-mediated control of CD8 T cell immune responses. Interestingly, tumor–specific CD8 T cells frequently accumulate in metastatic LNs and visceral tumor tissues from cancer patients, but are deficient in mediating cytolytic activity (10). The present data suggest that tumor–specific CD4 Treg may be responsible for the functional tolerance of the tumor–specific CD8 T cells. Further experiments are underway to address in more detail (i) the antigen–specificity of Tregs, and (ii) the sites of activation and action of Tregs *in vivo*.

Immunotherapy of patients with cancer requires *in vivo* generation of highly reactive tumor–specific T cells that are not restrained by normal tolerance mechanisms. Lymphodepletion has been recently reported to have a marked effect on the efficacy of adoptive transfer therapy of autologous tumor–specific T cells (7, 11). Here, we question whether such efficacy depends on the destruction of Treg or disruption of homeostatic T cell regulation.

In marked contrast with our previous findings, HA–specific CD8 T cells rejected HA^+ tumors, even in the presence of Treg, when cells were adoptively transferred into lymphodepleted hosts (Fig. 7). Furthermore, preliminary experiments also indicate potent *in vivo* cytolytic activity by HA–specific CD8 T cells in the lymphodepleted hosts, even in the presence of Treg (data not shown), confirming the importance of the cytolytic potential of the transferred CD8 T cells for successful tumor rejection.

We have also investigated the possibility that proliferation of Treg may by slower than that of CD8 T cells in the lymphodepleted hosts, and as a consequence, that the latter cells may no longer be suppressed *in vivo*. However, both CD8 T cells and Treg extensively divided in lymphodepleted hosts, and the ratios between Treg and CD8 T cell populations were similar in irradiated and nonirradiated hosts (data now shown). Alternatively, Treg might have lost their suppressor activity in the lymphodepleted hosts. Although we need to further address this issue, it has already been demonstrated in another experimental system that Treg remained fully functional in a lymphopenic environment (12).

We are currently exploring the possibility that the functional activity of CD8 T cells is simultaneously controlled by Treg and homeostatic mechanisms, which respectively suppress and promote CD8 T cell cytolytic activity. At this time, our data suggest that a homeostatic–induced activation overcomes suppression of cytolytic CD8 T cell activity

by Treg. Interestingly, preliminary observations indicate that naïve HA–specific CD8 T cells that undergo expansion in irradiated lymphopenic mice become resistant to regulation by CD4+CD25+ regulatory T cells, and proceed to reject transplanted tumors specifically (Figure 8). The CD8 T cells, when adoptively transferred into lymphodepleted, non-tumor bearing recipients, not only extensively proliferate but also gain the capacity to potently and specifically kill HA peptide–pulsed syngeneic splenocytes *in vivo* ; in a tumor bearing mouse, specific kill activity is restricted to the tumor draining lymph node (data not shown). Thus, HA–specific CD8 T cells may acquire strong and specific cytolytic effector functions through lymphopenic expansion *in vivo*. If confirmed, these observations are likely to significantly impact the design of future immunotherapies.

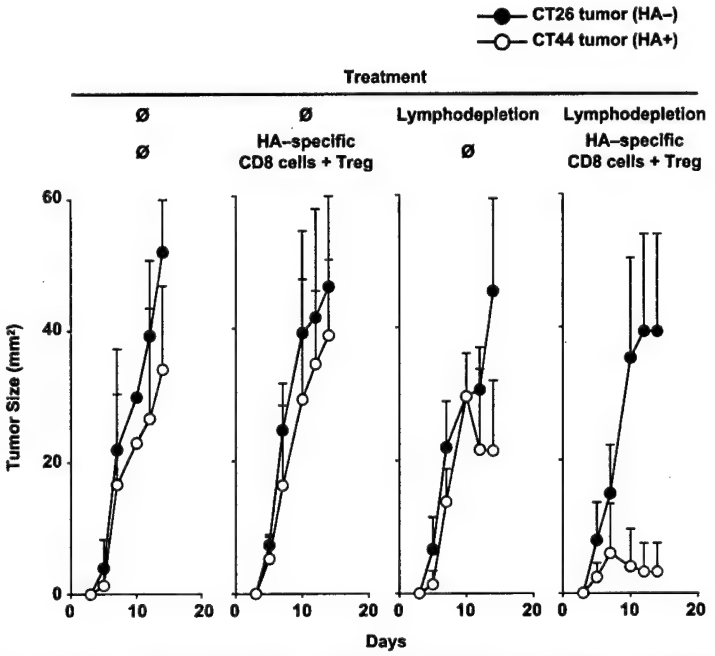

Figure 8. Lymphodepletion rescues the capacity of HA–specific CD8 T cells to reject HA–expressing tumors in the presence of Treg. Mice were subjected or not to irradiation (500 rad) (Day–2), were adoptively transferred or not with 10^5 HA–specific CD8 T cells and Treg (day–1), then were inoculated s.c. with 10^6 tumor cells (CT26 (HA–) tumor cells, let footpad, and CT44 (HA+) tumor cells, right footpad).

KEY RESEARCH ACCOMPLISHMENTS:

- $PGK^{loxP}lacZ^{loxP}HA$ transgenic mice were generated that expressed an ectopic antigen (influenza HA) in a Cre/loxP manner and were crossed with WAP-Cre mice. In contrast to $Catnb^{lox(ex3)}$or R26R mice, Cre/loxP mediated recombination in $PGK^{loxP}lacZ^{loxP}HA$ transgenic mice was not tissue specific but rather involved the germ line; this indicates that the frequency of recombination is strictly locus dependent.
- Expression of HA in the recombined $PGK^{loxP}lacZ^{loxP}HA$ mice led to the appearance of potent regulatory T cells, that suppressed active immune responses in an antigen dependent manner.
- Low level intra-thymic expression of HA was sufficient for the appearance of HA specific regulatory T cells; this observation has direct implications for anti tumor immune responses, as many tumor and tissue specific antigens are known to be expressed in low levels in the thymus.
- Adoptive transfer experiments using such regulatory T cells provided the unexpected result that the cells are (contrary to expectations) not anergic *in vivo*, but rather expand in response to vaccination to dominate the immune response to antigen.
- The mode of action of such regulatory T cells seems to be primarily to suppress the expansion of naïve CD4 T cells upon encounter of antigen. There was no evidence for immune deviation in the naïve T cell population.
- Surprisingly, antigen specific proliferation of CD8 T cells was not hindered by regulatory T cells, while their cytotoxic activity was lost.
- Preliminary observations indicate that in a lymphopenic environment the CD8 cells can recover their cytolytic activity and reject tumor; this observation may have significant impact on future design of immunotherapies of cancer.
- To extend these studies to autochtonous mammary tumors, new knock-in mice encoding the HA cassette in the ROSA-26 locus were generated and characterization of these mice is in progress. In parallel a second antigen model has been established that allows the follow up of immune responses against bacterial β-galactosidase. This model benefits from the availability of already characterized mice that express β-galactosidase (from the ROSA-26 locus) in a Cre dependent and tissue specific manner.

REPORTABLE OUTCOMES:

Klein, L., **Khazaie, K.,** von Boehmer, H. 2003 In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. **Proc Natl Acad Sci U S A**. 2003 Jul 22;100(15):8886-91.

Psarras, S., Karagianni, N., Kellendonk, C., Tronche, F., Cosset, F. L., Stocking, C., Schirrmacher, V., von Boehmer, H., **Khazaie, K.** 2004 Gene transfer and genetic modification of ES cells by Cre and Cre-PR expressing MESV based retroviral vectors. **Journal of Gene Medicine**. 6: 32-42.

Manuscript in preparation:
Chen, M.L., Pittet, M, von Boehmer, H., **Khazaie, K.** 2004 Suppression of tumor specific CD8 T cell cytolytic activity by CD4+CD25+ regulatory T cells. In preparation.

Conclusions

Cancer is likely to outdo the host immune responses at both the systemic and local levels. Future immune therapy strategies therefore have to consider strengthening the host at both levels. Action of antigen specific regulatory cells may provide a common mechanism for systemic tolerance, intended to protect against autoimmunity, but also preventing effective immune responses against tumors. We had reported that intrathymic expression of antigens lead to the generation of potent regulatory T cells. Our more recent efforts clearly show that these cells are capable of neutralizing otherwise effective cytolytic activities targeted against the tumor. The first stage of the battle is won, once the tumor specific regulatory cells are eliminated, or the tumor specific cytolytic cells are rendered resistant to regulation. Our work has provided preliminary evidence that the latter is indeed possible to achieve, with relatively simple manipulations of the host prior to adoptive T cell transfer, or possibly vaccination.

We have learned important technical lessons from the Cre/loxP based animal models generated so far in the course of this work. The choice of genetic locus for targeted recombination is critical. It appears that certain loci are significantly more susceptible than others for recombination. These are unsuitable for engineering tissue/tumor specific recombination, as they tend to promote germ-line recombination events. One suitable locus is the ROSA-26. Cre mediated recombination in this site is tightly controlled and when Cre is expressed in a tissue specific manner, the resulting recombination is tissue specific. We have therefore made knock-in mice, inserting our HA cassette in this locus, and have obtained mice that will allow us to follow responses against an alternative ectopic antigen (lacZ) targeted to this site and expressed in a Cre dependent manner.

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BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Khashayarsha Khazaie	Assistant Professor, Ph.D. D.Sc.

EDUCATION

INSTITUTION AND LOCATION	YEAR	DEGREE CONFERRED	FIELD OF STUDY
University of Surrey, Guildford, Surrey, U.K.	B.Sc.	1978	Medical Biochemistry
National Institute for Medical Research, London, U.K.	PhD	1982	Genetics/Aging
Rene Descartes University, Paris FR	D.Sc.	1998	Tumor Biology

ACADEMIC APPOINTMENTS

2003- Assistant Professor, Mol Imaging/Radiology, MGH, Harvard Medical School. Boston, MA
1999-2003 Instructor of Pathology, Harvard Medical School. Boston, MA
1998-1999 Visiting Faculty, University of Paris V Medical School, Université René Descartes, Paris, France.
1989-1998 Research Group Leader, Division of Tumor Immunology, German Cancer Research Center, Heidelberg, Germany.

HOSPITAL APPOINTMENTS

2002- Senior NRSA Fellow in Cancer Immunology, Dana Farber Cancer Institute.
1999-2003 Instructor, Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute.
1998-1999 Visiting Senior Scientist, National Institute of Science and Medicine Research, Department of Médecine Necker-Enfants Malades, Paris, France.

POSTDOCTORAL TRAINING

1982-1985 Research Fellow, Department of Gene Structure and Expression, National Institute for Medical Research, London, U.K.
1985-1987 Research Fellow, Differentiation Program, European Molecular Biology Laboratory Heidelberg, Germany.
1987-1989 Research Fellow, Laboratory of Molecular and Cellular Immunology Department of Medicine Lyon, France.

WORKSHOPS

October 1-5, 1999 Techniques for Modeling Human Mammary Cancer in Mice, The Jackson Laboratory, Bar Harbor.
October 20-29, 2000 Modeling Human Colo-Rectal Cancer in Mice, The Jackson Laboratory, Bar Harbor.
April 3-9, 2002 Mechanisms and Applications of Immune Tolerance, Keystone Symposium, Steamboat, Colorado

ORIGINAL REPORTS:

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2) **Khazaie, K.**, Buchanan, J., and Rosenberger, R. 1984. The accuracy of Qβ RNA translation: 2. Errors during the synthesis of proteins by cell free *Escherichia coli* extracts. **Eur. J. of Biochem.** 144, 491-495.
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Vennstrom, B., Beug,, H., Forrest, D., Johnson, A., **Khazaie, K.**, Munoz, A., Sap., Ullrich, A., Zenke, M. 1989. Functions of the erbA and erbB oncogenes in avian erythroblastosis. NATO ASI Series, Vol. H26, *Cell to Cell Signals in Mammalian Development*, Edited by S.W. de Laat et al., Springer-Verlag Berlin Heidelberg.

Zenke, M., **Khazaie, K.**, Beug, H. 1990. V-myc transformed macrophages expressing the normal human EGF receptor are induced to proliferate by EGF via a nonautocrine mechanism. In *Molecular Biology of Hematopoiesis*, Edited by L. Sachs, N.G. Abraham, C.J. Wiedermann, A.S. Levine, G. Konwalinka, pp 453-467. Intercept, Andover, Hampshire, Great Britain.

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Chlichlia, K., Los, M., Schulze-Osthoff, L., Gazzolo, L., Schirrmacher, V., **Khazaie, K.** 2002. Redox events in HTLV-I Tax-induced apoptotic T-cell death. *Antioxidants and Redox Signaling (ARS)*. Vol. 4, Nr. 3, 471-477.

Cassens, U., Lewinski, G., Samraj, A.K., von Bernuth, H., Baust, H., **Khazaie, K.** and Los, M. 2002. Viral Modulation of Cell Death by Inhibition of Caspases. . *Arch. Immunol. Ther. Exp.*, 51, 19-27.

Research projects completed or ongoing in the last three years (PI: K. Khazaie)

APPENDIX 2

Ongoing Research Support (PI: K. Khazaie)

Idea Award. May 2002 - May 2005
Department of Defense Breast Cancer Research Program,
Title: Cancer Immunology in an inducible model of breast cancer.
Major goals: To develop an animal model of inducible mammary cancer, and to use this model to study antigen specific immune responses against the mammary gland and mammary tumors.

Idea Award. 2003-2008
Department of Defense Prostate Cancer Research Program,
Title: Initiating events in prostate cancer: The role of somatic activation of β-catenin.
Major goals: To evaluate the stabilization of β-catenin as an initiating event in prostate cancer.

Senior National Research Council Award Jan 2003 – Jan 2005
National Cancer Institute
(Salary Support only)

Completed Research Support
Inter-programmatic Research Award. 2002-2004
Dana Farber Cancer Institute/Harvard Cancer Center
Title: An animal model for investigating immunosurveillance and immunotherapy in prostate cancer.
Major goals: To develop an animal model of prostate cancer based on the prostate specific activation of the APC/β-catenin pathway, and to use this model to study antigen specific immune responses against the prostate.

National Colorectal Cancer Research Alliance. (Award) 2001
Entertainment Industry
Title: An Animal Model for Designing Targeted Immune Intervention in Colon Cancer.
Major goals: To Investigate antigen specific immune responses in the healthy and neoplastic mouse intestine.

Hershey Prostate Cancer/Survivors Walk. (Award) 2001
Beth Israel Hospital
Title: somatic activation of β-catenin reveals a critical event in the initiation of prostate cancer.
Major goals: To Investigate the role of β-catenin in prostate cancer.

Applied for
Claudia Adams Barr Program in Cancer Research. Boston, MA.
Dana Farber Cancer Institute
Title: Mast cells. And orchestration of local inflammatory reactions in colon cancer.
Major goals: To investigate the cross-talk between tumor epithelium and infiltrating mast cells.

RO1 submitted in 2003
Title: Imaging Proteolytic Activity in Colon Cancer
Major goals: Image proteolytic activity in animal models of colon cancer, and apply imaging to detection of tumor status and biological response.

RO1 submitted in Feb 2004
Title: Inflammation in Colon Cancer: A Cause or consequence?
Major goals: Define the role of innate immune response in the initiation of polyposis and in progression of invasive carcinomoma.

In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior *in vitro*

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Adoptive transfer of antigen-specific CD25$^+$CD4$^+$ regulatory T cells was used to analyze the stability of their phenotype, their behavior after immunization, and their mode of suppressing cotransferred naive T cells *in vivo*. We found that regulatory T cells maintained their phenotype in the absence of antigen, were not anergic *in vivo*, and proliferated as extensively as naive CD4$^+$ T cells after immunization without losing their suppressive function *in vivo* and *in vitro*. *In vivo*, the expansion of cotransferred naive T cells was suppressed relatively late in the response such that regulatory T cells expressing mostly IL-10 but not IL-2 or IFN-γ represented the dominant subset of cells. Our results reveal properties of regulatory T cells that were not predicted from *in vitro* studies.

Dominant mechanisms of tolerance control the autoimmune potential of self-reactive T cells in healthy individuals and animals (reviewed in refs. 1–3). Insights into the regulation of immune responses by regulatory T cells have been mostly obtained with polyclonal populations of regulatory T cells for which the role of specific antigen has been largely obscure. The impact of self-antigen on the shaping of the regulatory T cell pool came more into focus when it was observed that the coexpression of a transgenic class II MHC restricted T cell receptor (TCR) and its agonist ligand resulted in the generation of antigen-specific regulatory T cells (4). Subsequent experiments in that system confirmed the notion that thymic epithelium can have a decisive role in the formation of such cells by demonstrating that the expression of an agonist ligand on radioresistant tissue (5) and on transplanted thymic epithelium (6) was a very effective means of generating regulatory T cells, whereas the mode of generation of polyclonal regulatory T cells in normal mice still needs to be elucidated.

A subset of CD4$^+$ T cells expressing the interleukin-2 (IL-2) receptor α-chain (CD25) recently became a major focus of interest. These CD4$^+$CD25$^+$ T cells were first shown by Sakaguchi and colleagues to control autoreactive T cells *in vivo* (7). Several characteristics of these cells have emerged from *in vitro* studies (reviewed in ref. 8) resulting in the notion that regulatory T cells are anergic in terms of proliferation and suppress other cells by direct cell contact, which requires neither IL-10 nor transforming growth factor-β and which results in the inability of suppressed CD4$^+$ T cells to produce IL-2 (9–11). However, it is unclear at present how far these observations *in vitro* are in fact a reflection of the properties of CD4$^+$CD25$^+$ T cells *in vivo*. In some experimental systems of immune regulation by CD4$^+$CD25$^+$ T cells *in vivo*, it was found that soluble factors such as IL-4, IL-10, and transforming growth factor-β do contribute to the prevention of autoimmunity, with the role of these factors varying between different models (12–14). Polyclonal CD25$^+$CD4$^+$ T cells proliferate and expand when they are transferred into *rag*$^{-/-}$ or IL-2 receptor β-deficient mice (15–17), indicating that their anergic state can be reversed under certain nonphysiological conditions. Notably, it is under exactly these lymphopenic conditions that the regulatory function of CD25$^+$CD4$^+$ T cells has been studied in the majority of the currently available models, at least suggesting that proliferation and suppressive function by regulatory T cells may not be mutually exclusive. A major experimental drawback of lymphopenic models of immune regulation, however, is that they provide no information on antigen-induced proliferation as observed in nonlymphopenic mice.

The present study was initiated to establish an *in vivo* system of antigen-specific immune regulation that is as physiological as possible to characterize the behavior of CD25$^+$CD4$^+$ regulatory T cells at relatively low frequency in the context of an unperturbed immune system. To this end, TCR transgenic CD25$^+$CD4$^+$ T cells were adoptively transferred into normal hosts either alone or in combination with naive CD4$^+$25$^-$ T cells of identical antigen specificity. Immunization with cognate antigen was used to visualize and compare the behavior of the respective populations either alone or in combination.

Materials and Methods

Mice and Transgenic Vectors. BALB/c Thy1.2 mice were purchased from Taconic Farms. Other strains were bred in the animal facility of the Dana–Farber Cancer Institute under specific pathogen-free conditions.

Phosphoglycerate kinase-hemagglutinin (*pgk-HA*) mice were generated through germ-line cre-mediated recombination in mice carrying a *pgk-*^{lox}*β-gal*^{lox}*-HA* cassette vector. This vector was constructed as follows. Lox P sites were introduced 5′ and 3′ of the *β-gal* cDNA. The HA cDNA then was introduced downstream of the 3′ lox P site, and the construct was cloned into a eukaryotic expression vector driven by the *pgk* promoter. Transgenic founders (F_1 FVB × 129) were backcrossed to BALB/c for at least five generations before being crossed to TS4 cre mice (18) or Whey acidic protein-cre mice (19) that had been backcrossed to BALB/c for at least four generations. Both crosses unexpectedly led to germ-line recombination of the transgene. Germ-line-recombined mice then were maintained by further backcrossing of *pgk-HA* × *TCR-HA* double-transgenic mice to BALB/c for at least four generations.

Immunization. Mice were immunized s.c. with 100 μg of peptide HA$^{107–119}$ emulsified in incomplete Freund's adjuvant (IFA, Sigma–Aldrich). At the indicated time points the animals were killed, and draining (popliteal and inguinal) and distant (mesenteric) lymph nodes were harvested for analysis. The tumor cell line CT26 HA-EGFP has been described elsewhere (20).

Antibodies and Fluorescence-Activated Cell Sorter Analysis. Biotin-conjugated mAbs to CD4 (H129.19) and Thy1.1 (HIS51), phycoerythrin-conjugated mAbs to CD4 (GK1.5), CD25 (PC61), IL-2 (JES6–5H4), IL-10 (JES5-16E3), IFN-γ (XMG.1.2), tumor necrosis factor α (MP6-XT22), rat IgG$_1$ isotype control (R3-34), and rat IgG$_{2b}$ isotype control (A95-1), Cy-chrome-conjugated streptavidin, mAbs to CD8 (53-6.7), and allophycocyanin-conjugated mAbs to CD4 (RM4-5), CD25 (PC61), and Thy1.2

Abbreviations: TCR, T cell receptor; pgk, phosphoglycerate kinase; HA, hemagglutinin; IFA, incomplete Freund's adjuvant; CFSE, 5,6-carboxyfluorescein diacetate-succinimidyl ester.

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(53-2.1) were purchased from Becton Dickinson. The mAb to the *TCR-HA* (6.5) was purified and conjugated with FITC in our lab. Fc-receptor-blocking mAb 2.4G2 was used as culture supernatant. Surface stainings were performed according to standard procedures at a density of 2–4 × 10^6 cells per 50 μl, and volumes were scaled up accordingly. Flow-cytometric analysis was performed on a FACSCalibur (Becton Dickinson) by using CELLQUEST software (Becton Dickinson).

Intracellular Cytokine Staining. Cells from the draining lymph node of immunized animals were resuspended at a density of 2 × 10^6 cells per ml in Iscove's modified Dulbecco's medium containing 10% FCS. Cells then were plated in 6 ml per well into six-well culture plates, 12 μl of leukocyte activation mixture with GolgiPlug (Becton Dickinson) containing phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A were added, and cultures were incubated at 37°C for 6 h. Cells were harvested and incubated with 2.4G2 Fc-receptor-blocking antibody before surface staining. Cells then were fixed with Cytofix/Cytoperm buffer (Becton Dickinson) for 20 min at room temperature. Intracellular cytokine staining was performed at room temperature for 20 min in Perm/Wash buffer (Becton Dickinson). Control stainings were performed with a mixture of phycoerythrin-conjugated isotype controls.

Purification and Adoptive Transfer of Cells. Pooled cells from spleen and peripheral lymph nodes (mesenteric, axillary, brachial, popliteal, inguinal, and cervical) from *pgk-HA* × *TCR-HA* mice were subjected to erythrocyte lysis. Cells then were incubated with Fc-receptor-blocking antibody 2.4G2 and stained with anti-CD4 biotin. After incubation with streptavidin microbeads (Miltenyi Biotec, Auburn, CA), $CD4^+$ cells were positively selected on midi-MACS columns, routinely achieving purities >95%. Cells then were stained with streptavidin-Cy-chrome, anti-CD25 phycoerythrin, and FLUOS-labeled 6.5. $CD4^+CD25^+6.5^+$ cells were sorted by using a MoFlow cell sorter (Cytomation, Fort Collins, CO). Naive *TCR-HA* $CD4^+$ T cells were obtained from spleen and lymph nodes of *TCR-HA* $rag^{-/-}$ mice by magnetic enrichment for CD4 T cells.

Cells were injected into the lateral tail vein in a volume of 200 μl of PBS. Where indicated, cells were labeled with 5,6-carboxyfluorescein diacetate-succinimidyl ester (CFSE) (Molecular Probes) by incubation for 10 min at 37°C in 10 μM CFSE in PBS/0.1% BSA at a density of 1 × 10^7 cells per ml.

Proliferation Assays. For inhibition assays, 2 × 10^4 sorted or magnetically enriched $CD25^+$ and/or $CD25^-$ $CD4^+6.5^+$ T cells were incubated with 2 × 10^5 irradiated (3,000 rad) BALB/c splenocytes in the presence of 5 μg/ml $HA^{107-119}$ peptide in 200 μl of Iscove's modified Dulbecco's medium supplemented with 10% FCS in 96-well round-bottom plates. Where indicated, 50 units/ml recombinant IL-2 were added (Becton Dickinson). Proliferation was measured by scintillation counting after pulsing with 1 μCi per well [^{3}H]thymidine (1 Ci = 37 GBq) for the last 16–20 h of a 90-h incubation period.

For *ex vivo* proliferation assays, cells from draining lymph nodes were cultured for 72–90 h in triplicates at 4 × 10^5 cells per well in round-bottom 96-well plates in serum-free medium (HL-1, BioWhittaker). Proliferation was measured as incorporation of [^{3}H]thymidine, which was added for the last 18 h of culture (1 μCi per well).

Results

High Frequency of Antigen-Specific $CD25^+CD4^+$ Regulatory T Cells in *pgk-HA* × *TCR-HA* Mice. Mice expressing influenza-HA under the control of the ubiquitous *pgk* promoter, in the following referred to as *pgk-HA* mice, were crossed to mice expressing a transgenic TCR (*TCR-HA*) specific for peptide 111–119 of HA (21). Among

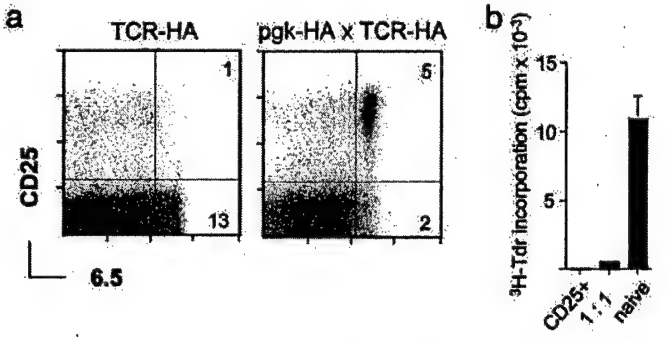

Fig. 1. High frequency of HA-specific regulatory $CD4^+CD25^+$ T cells in *pgk-HA* × *TCR-HA* mice. (*a*) Expression of the transgenic TCR (mAb 6.5) versus CD25 on gated CD4 T cells from lymph nodes of *TCR-HA* single-transgenic versus *pgk-HA* × *TCR-HA* double-transgenic mice. Numbers in the dot plots indicate the percentage of gated cells within the respective quadrants. (*b*) $CD25^+$ CD4 T cells from *pgk-HA* × *TCR-HA* mice were anergic and suppressed the proliferation of naive CD4 T cells from *TCR-HA* $rag^{-/-}$ mice *in vitro*. Sorted $CD4^+CD25^+6.5^+$ cells from *pgk-HA* × *TCR-HA* mice and naive $CD4^+6.5^+$ T cells from *TCR-HA* $rag^{-/-}$ mice were incubated either alone or together (ratio 1:1) in the presence of BALB/c splenocytes and HA-peptide for 90 h. Proliferation was measured as incorporation of [^{3}H]thymidine (^{3}H-Tdr) added for the last 20 h.

lymph node cells of *pgk-HA* × *TCR-HA* mice, the fraction of $CD4^+$ $TCR\text{-}HA^+$ cells was reduced by a factor of 2 as assessed by staining with the anti-clonotypic antibody 6.5, and a distinct population of 6.5^+CD25^+ cells that could not be detected in TCR single transgenics was observed (Fig. 1*a*). Sorted 6.5^+CD25^+ cells from thymus (Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org) or periphery (Fig. 1*b*) of double-transgenic mice were anergic *in vitro* and suppressed the proliferation of naive 6.5^+CD4^+ T cells in standard coculture assays. The addition of IL-2 enabled proliferation of 6.5^+CD25^+ cells from both thymus and periphery (data not shown). Thymus transplantation revealed that expression of HA by radioresistant thymic epithelium was sufficient to mediate the selection of 6.5^+CD25^+ cells (data not shown). Thus, by these criteria, the HA-specific $CD25^+$ cells were equivalent to polyclonal $CD25^+$ suppressor T cells from normal mice.

Suppression of Anti-HA Responses *in Vivo* After Transfer of $CD25^+$ Regulatory T Cells. 6.5^+CD25^+ CD4 T cells from *pgk-HA* × *TCR-HA* $Thy1.2^+$ mice were transferred into $Thy1.1^+$ hosts. The frequency of donor-derived cells among host cells was similar when equal numbers of either $CD25^+$ cells from *pgk-HA* × *TCR-HA* mice or naive 6.5^+CD25^- cells from *TCR-HA* $rag^{-/-}$ mice were transferred. Fourteen days after transfer, most (>85%) donor-derived cells still expressed the CD25 marker, and their number appeared stable within this time frame (data not shown). Reisolated cells were still anergic and suppressed naive 6.5^+ CD4 T cells *in vitro* (Fig. 2 *a* and *b*), indicating that they represented a lineage rather than antigen-dependent effector cells.

We next asked whether transferred 6.5^+CD25^+ cells could inhibit the response of endogenous HA-specific T cells. Recipients of 3 × 10^5 6.5^+CD25^+ cells, corresponding to a frequency of approximately 1 in 3,000 $CD4^+$ T cells, or noninjected control mice were immunized with peptide $HA^{107-119}$ in IFA. Although draining lymph node cells from control animals proliferated vigorously when restimulated *in vitro*, no proliferation was detected with cells from recipients of regulatory T cells (Fig. 2*c*).

The colon carcinoma cell line CT26-HA, which stably expresses HA (20), was used to test whether transferred 6.5^+CD25^+ cells influenced the growth of an HA-expressing

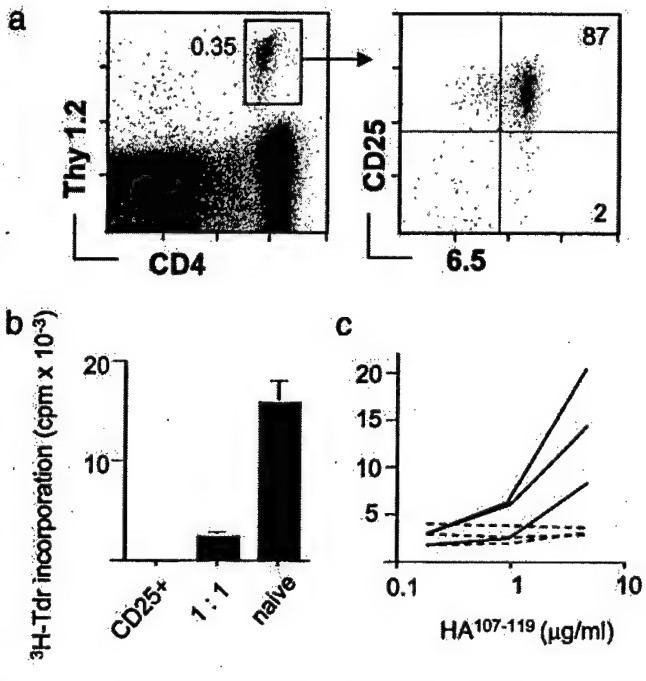

Fig. 2. Transferred CD4$^+$CD25$^+$6.5$^+$ T cells retain their phenotypic and *in vitro* regulatory properties in the absence of antigen and suppress endogenous anti-HA-specific T cells after immunization. (*a*) CD4$^+$CD25$^+$6.5$^+$ T cells (4×10^6) from Thy1.2$^{+/+}$ *pgk-HA* × *TCR-HA* mice were transferred into BALB/c Thy1.1$^{+/+}$ mice. Six days after transfer, the recipients were killed. Peripheral lymph nodes and spleen were pooled and stained for CD4, Thy1.2, CD25, and 6.5. The frequency of donor-derived cells among CD4 T cells is shown together with the sorting gate used for reisolation of cells. (*Right*) Purity of reisolated cells. (*b*) Reisolated Thy1.2$^{+/+}$ cells 6 days after transfer were tested for their proliferative response after stimulation with HA-peptide and their suppressive potential when cocultured with naive cells as described for Fig. 1. (*c*) Recipients of 3×10^5 CD4$^+$CD25$^+$6.5$^+$ T cells (dashed lines) or untreated BALB/c mice (solid lines) were immunized with HA-peptide (100 μg) in IFA. Eight days later, draining lymph node cells were harvested and stimulated *in vitro* with titrated amounts of HA-peptide for 90 h. Incorporation of [^{3}H]thymidine (^{3}H-Tdr) within the last 20 h was measured. The graph shows the data for three immunized mice of each group representative for three independent experiments.

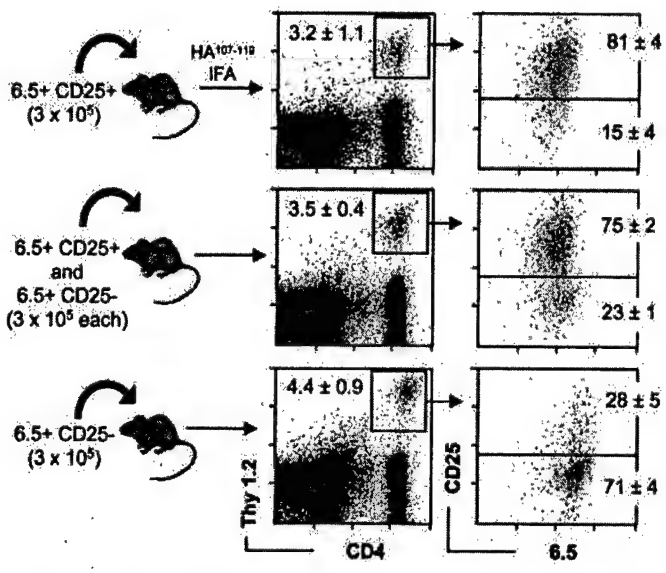

Fig. 3. Accumulation of CD4$^+$CD25$^+$6.5$^+$ T cells or naive CD4$^+$CD25$^-$6.5$^+$ T cells in the draining lymph nodes after immunization. (*a*) Thy1.1$^{+/+}$ recipients of CD4$^+$CD25$^+$6.5$^+$ T cells, CD4$^+$CD25$^-$6.5$^+$ T cells, or both types of cells were immunized with HA-peptide as described for Fig. 2. Draining lymph node cells were harvested on day 8 after immunization and stained for CD4, Thy1.2, CD25, and 6.5. (*Left*) Numbers in the dot plots indicate the percentage of donor-derived cells among CD4$^+$ T cells (mean of four animals). (*Right*) The dot plots show the expression of the transgenic TCR (6.5) versus CD25 on gated CD4$^+$Thy1.2$^+$ cells. Numbers within the dot plots indicate the percentage of gated cells in the respective quadrant (mean of four animals).

tumor by suppressing the antitumor response. Subcutaneous inoculation of normal BALB/c mice with CT26-HA leads to the induction of anti-HA CD4 and CD8 T cell responses (20). Transfer of 6.5$^+$CD25$^+$ cells allowed for accelerated growth of the tumor (Fig. 8, which is published as supporting information on the PNAS web site). Furthermore, although in tumor-bearing normal mice or mice that had received naive CD25$^-$ 6.5$^+$ CD4 T cells a strong *ex vivo* proliferative response was observed, such a recall response was absent with cells from recipients of CD25$^+$ 6.5$^+$ T cells irrespective of whether naive cells had been cotransferred (Fig. 8). Thus, transferred CD25$^+$ cells had potent suppressive activity *in vivo*.

Antigen-Driven Expansion of CD25$^+$CD4$^+$ T Cells *in Vivo*. We next aimed to visualize and compare the behavior of antigen-specific regulatory and naive T cells after immunization. A control group received 3×10^5 6.5$^+$CD25$^-$ cells only and was immunized the following day. On day 8 after immunization, ≈4% of draining lymph node CD4 T cells were donor-derived, and the majority of these cells was CD25$^-$ (Fig. 3). In nondraining lymphoid compartments, <0.05% of CD4 T cells were Thy1.2$^+$, similar to the values observed in nonimmunized recipients (data not shown). Thus, HA-specific "naive" T cells expectedly increased ≈100-fold in the antigen-exposed lymph node. A second group of animals received 3×10^5 6.5$^+$CD25$^+$ cells and was immunized and analyzed as described above. Surprisingly, we found a distribution of donor-derived cells in these animals very similar to that observed in recipients of naive cells. Approximately 3% of CD4 T cells in the draining lymph nodes were donor-derived, whereas in distant lymphoid compartments, <0.05% of CD4 T cells were Thy1.2$^+$ (data not shown). Contrary to recipients of naive cells, these cells mostly expressed the CD25 marker (Fig. 3). A third group received both 6.5$^+$CD25$^+$ and naive 6.5$^+$CD25$^-$ cells. Eight days after immunization the proportion of donor-derived cells among CD4 T cells in the draining lymph node was again ≈3%, and the majority of these cells expressed high levels of CD25, as was observed in mice that had received 6.5$^+$CD25$^+$ cells only (Fig. 3).

The almost identical frequencies of donor-derived cells in the draining lymph nodes of recipients of either regulatory or naive T cells implied a similar homing/expansion pattern of both cell types, a surprising finding considering the absence of an *in vitro* recall response in recipients of CD25$^+$ T cells (see Fig. 2*c*). To confirm this apparent discrepancy, we restimulated draining lymph node cells from the three groups with HA-peptide *in vitro*. Cells from recipients of naive cells showed a very strong response, whereas neither recipients of 6.5$^+$CD25$^+$ cells alone nor recipients of mixed populations displayed any antigen-specific *in vitro* proliferation (data not shown).

To address whether antigen-driven expansion *in vivo* altered the properties of 6.5$^+$CD25$^+$ cells *in vitro*, we sorted these cells from draining lymph nodes of immunized recipients and compared them with 6.5$^+$CD25$^+$ cells taken directly from *pgk-HA* × *TCR-HA* mice. When stimulated *in vitro*, both populations were completely anergic with respect to proliferation, and provision of IL-2 restored some proliferation in both types of cultures, yet less efficiently with "expanded regulators" (data not shown). When titrated in the standard coculture assay, expanded regulators were ≈4-fold more efficient suppressors than "nonexpanded

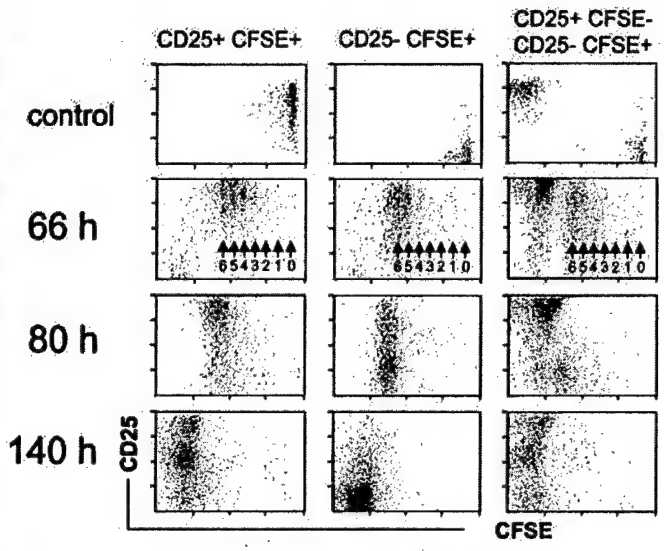

Fig. 4. Proliferation of adoptively transferred $CD4^+CD25^+6.5^+$ T cells or $CD4^+CD25^-6.5^+$ T cells in the draining lymph nodes after immunization. CFSE-labeled $CD4^+CD25^+6.5^+$ T cells from *pgk-HA* × *TCR-HA* mice (3×10^5) (*Left*) or CFSE-labeled naive $CD4^+CD25^-6.5^+$ T cells from *TCR-HA rag*$^{-/-}$ mice (3×10^5) were transferred into BALB/c Thy1.1 mice. Two days later, recipients were immunized with 100 μg of HA-peptide in IFA. Controls were immunized with IFA without peptide. Mice were killed at the indicated time points after immunization, and draining lymph node cells were harvested and stained for CD4, Thy1.2, and CD25. The dot plots show the expression of CD25 versus CFSE fluorescence intensity on gated donor-derived cells ($CD4^+Thy1.2^+$). Numbered arrows within the dot plots (66 h) indicate the number of divisions of CFSE-labeled cells. Note that all dot plots (except controls, where ~200 events are shown) show 800–1,000 $CD4^+Thy1.2^+$ events and thus do not represent the frequency of these cells among host CD4 T cells. (*Right*) Representative analysis of a cotransfer of 3×10^5 CFSE-labeled naive $CD4^+CD25^-6.5^+$ T cells from *TCR-HA rag*$^{-/-}$ mice and 3×10^5 unlabeled $CD4^+CD25^+6.5^+$ T cells from *pgk-HA* × *TCR-HA* mice into BALB/c Thy1.1 recipients.

regulators" (Fig. 9, which is published as supporting information on the PNAS web site).

$CD25^+$ Regulatory T Cells Do Not Affect the Initial Expansion of Naive Cells After Immunization. To address whether the accumulation of 6.5^+CD25^+ cells in the draining lymph nodes was a result of proliferation rather than homing, we labeled Thy1.2 6.5^+CD25^+ cells with CFSE before adoptive transfer. A control group received CFSE-labeled naive 6.5^+CD25^- cells. Animals were immunized as described before, and the phenotype of donor-derived cells in the draining lymph nodes was followed in a kinetic fashion by gating on $CD4^+Thy1.2^+$ cells. As early as 66 h after immunization, the majority of donor-derived cells in the draining lymph nodes of both groups of animals had cycled more than four times (Fig. 4). The progeny of 6.5^+CD25^+ cells had further up-regulated CD25, and CD25 was also expressed by the progeny of naive cells at this point in time, as expected. Eighty hours after immunization, cells in both groups of animals had undergone further divisions, the exact numbers of division no longer being discernable. The progeny of 6.5^+CD25^+ as well as of 6.5^+CD25^- cells appeared to divide in a "synchronized" wave (Fig. 4), probably indicating that the immunization protocol induced a transient window of productive antigen presentation in the draining lymph node. Donor-derived cells in the draining lymph nodes of both types of recipients had lost their CFSE-label 140 h after immunization. The progeny of 6.5^+CD25^+ cells contained cells that had returned to initial levels of CD25 expression, whereas others maintained elevated levels. By contrast, the progeny of naive $CD25^-$ cells had mostly lost expression of CD25. Both findings were in accord with our previous observation (compare with Fig. 3).

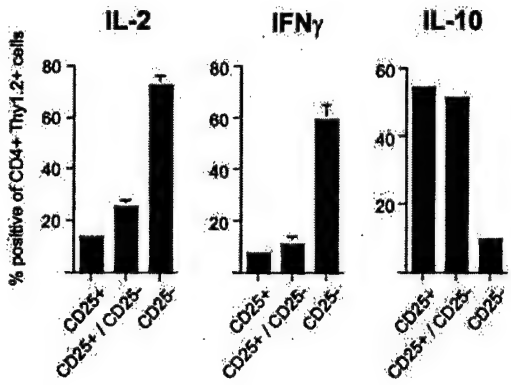

Fig. 5. Cytokine production of transferred $CD4^+CD25^+6.5^+$ T cells or $CD4^+CD25^-6.5^+$ T cells. (*a*) Transferred into Thy1.2 recipients were 3×10^5 $CD4^+CD25^+6.5^+$ T cells, $CD4^+CD25^-6.5^+$ T cells, or both. Mice were immunized with HA-peptide in IFA, and draining lymph node cells were harvested 8 days after immunization. Cells were restimulated *in vitro* with phorbol 12-myristate 13-acetate/ionomycin for 6 h in the presence of brefeldin A before surface staining for CD4 and Thy1.2, fixation, and intracellular staining for the indicated cytokine. The frequencies of cytokine-positive cells among gated $CD4^+Thy1.2^+$ draining lymph node cells of the indicated groups of animals are shown (for original data see Fig. 10).

We next performed cotransfer experiments with regulatory and CFSE-labeled naive T cells (Fig. 4 *Right*). No major difference in the early (66-h) pattern of division was observed as compared with transfer of naive cells alone. Later, when the CFSE label was lost, it was again obvious that the progeny of regulatory cells, as identified by the $CD25^+$ or $CD25^{++}$ phenotype, represented the predominant donor-derived cell type in the draining lymph node (Fig. 4, compare with Fig. 3).

Inverse Cytokine Profile of Expanded Regulators and Activated Naive CD4 T Cells. The production of cytokines by HA-specific CD4 T cells under the different conditions was tested. Three groups of Thy1.1 animals were injected with $Thy1.2^+6.5^+CD25^+$ T cells, $Thy1.2^+6.5^+CD25^-$ naive T cells, or both, and animals were immunized as described before. Eight days later, cytokine production by donor-derived cells was determined after brief restimulation *in vitro*. In recipients of 6.5^+CD25^- naive cells only, a large fraction (>60%) of $Thy1.2^+$ cells produced IL-2 and IFN-γ, whereas among the progeny of 6.5^+CD25^+ cells (expanded regulators) only few cells produced either cytokine (Fig. 5 and Fig. 10, which is published as supporting information on the PNAS web site). By contrast, a high proportion of cells produced IL-10. Among cells derived from mice that had received equal numbers of regulatory and naive HA-specific CD4 T cells, the cytokine profile closely resembled that seen in recipients of regulatory T cells alone, again suggesting that the regulatory T cells dominated the response after the 8-day period.

Regulatory $CD25^+$ T Cells Suppress the Late Expansion of Naive CD4 T Cells. To visualize more conclusively the influence of regulatory T cells on naive cells at different phases, we adoptively transferred CFSE-labeled $Thy1.1^+6.5^+CD25^-$ T cells into Thy1.2 recipients that had either received $Thy1.2^+6.5^+CD25^+$ regulatory T cells or not. Gating on $Thy1.1^+$ CD4 T cells then was used to analyze the expansion of the naive T cells after immunization. The initial recruitment of cells into the response, their proliferation rate, and the early expansion up to 90 h after the immunization were almost identical in both groups (Fig. 6 *a* and *b*, compare with Fig. 4). At 90 h after immunization, however, a plateau in the number of $Thy1.1^+$ cells among CD4 T cells was

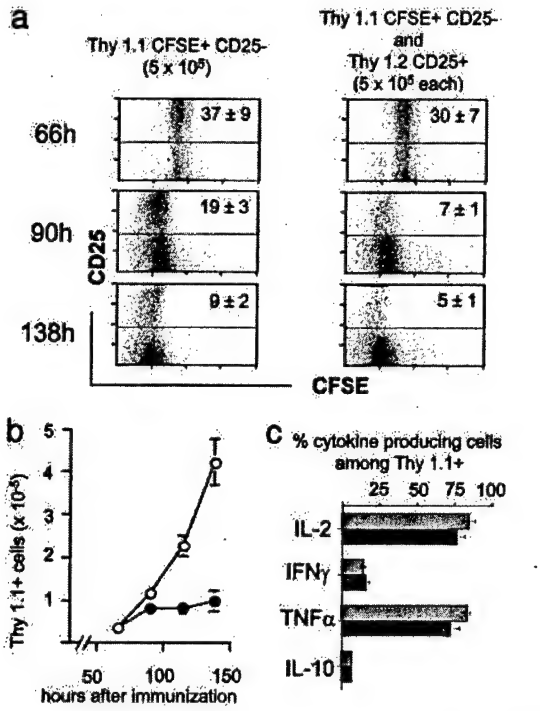

Fig. 6. Expansion, CD25 expression, and cytokine production after immunization of adoptively transferred naive $CD4^+CD25^-6.5^+$ T cells in the presence or absence of $CD4^+CD25^+6.5^+$ regulatory T cells. CFSE-labeled naive $CD4^+CD25^-6.5^+$ T cells (5×10^5) sorted from Thy1.1$^+$ *TCR-HA* mice (*rag*$^{+/+}$) were adoptively transferred into BALB/c Thy1.2 recipients (*Left*) or BALB/c Thy1.2 recipients that had in addition received an equal number of $CD4^+CD25^+6.5^+$ T cells from Thy1.2$^+$ *pgk-HA* × *TCR-HA* mice (*Right*). Mice were immunized as described before, and draining lymph node cells were harvested at the indicated time points after immunization. (*a*) Expression of CD25 versus CFSE fluorescence intensity on gated ($CD4^+$Thy1.1$^+$) progeny of naive $CD4^+CD25^-6.5^+$ T cells. Numbers within the upper quadrants indicate the frequency of $CD25^+$ cells among $CD4^+$Thy1.1$^+$ cells (mean of four per group). Note the "smearing" into lower-division numbers in the presence of regulatory T cells (*Right*). (*b*) Absolute number in the draining lymph nodes of the progeny of naive $CD4^+CD25^-6.5^+$ T cells after immunization in the presence (filled circles) or absence (open circles) of $CD4^+CD25^+6.5^+$ regulatory T cells (mean of four per group). The number at 0 h (i.e., without immunization) was $<0.1 \times 10^5$. (*c*) Draining lymph node cells were harvested on day 8 after immunization, stimulated *in vitro* with phorbol 12-myristate 13-acetate/ionomycin for 6 h in the presence of brefeldin A, and stained for CD4, Thy1.1, and the respective cytokine as indicated. The frequency of cytokine-producing cells among gated Thy1.1$^+$CD4$^+$ T cells is shown. Gray bars, recipients of naive $CD4^+CD25^-6.5^+$ T cells alone; black bars, recipients of naive $CD4^+CD25^-6.5^+$ T cells plus $CD4^+CD25^+6.5^+$ regulatory T cells (mean of four per group). TNFα, tumor necrosis factor α.

reached in animals that had received regulatory T cells (Fig. 6*b*). In contrast, in the absence of regulatory T cells the progeny of naive cells further expanded. Notably, even in the presence of regulatory T cells the majority of Thy1.1$^+$ cells continued to cycle between 90 and 138 h (Fig. 6*a*), although their number did not increase any further. Also, in the presence of regulatory T cells, a small yet distinct fraction of cells appeared to fall behind the bulk of dividing cells, discernable as a "smear" into higher CFSE intensities. Interestingly, at all time points analyzed, the fraction of cells among the progeny of naive cells expressing CD25 was lower in the presence of regulatory T cells (Fig. 6*a*).

We determined the cytokine production 8 days after immunization by Thy1.1$^+$ CD4 T cells from the two groups of mice, i.e., in either normally expanded or "suppressed" progeny of naive $CD25^-$ cells (Fig. 6*c*). The fraction of producers of IL-2, IL-10, IFN-γ, or tumor necrosis factor α was not affected significantly by suppression of their expansion through the presence of regulatory T cells, which indicated that indeed dominant expansion of regulatory T cells rather than "immune deviation" was the explanation for the observations depicted in Fig. 5.

Discussion

Most of our current understanding of the biology of suppressor T cells is derived from lymphopenic *in vivo* models or has been derived from the standard *in vitro* assay. Both types of systems have inherent limitations because of the abnormal behavior of lymphocytes in a lymphopenic environment (sometimes incorrectly referred to as homeostatic proliferation) and the questionable significance of *in vitro* observations in general. Our aim was to establish a model in which the behavior of antigen-specific $CD4^+CD25^+$ regulatory T cells and their influence on naive T cells after antigenic stimulation could be visualized *in vivo* in the context of an unperturbed immune system.

Within the time frame analyzed, the phenotype and function of $6.5^+CD25^+CD4^+$ T cells was stable after transfer into an antigen-free host. After immunization, $6.5^+CD4^+CD25^+$ T cells, despite their anergy *in vitro*, accumulated in the draining lymph nodes very much like naive T cells. CFSE labeling demonstrated that this accumulation was due to proliferation rather than preferential retention at the antigen-exposed site. These data are different from observations made with $CD25^+$ and $CD25^-$ regulatory T cells from mice that express high levels of antigen on hematopoietic cells (6), where persistent exposure to antigen may render regulatory T cells incapable of *in vivo* proliferation.

When reisolated, expanded regulators were anergic and exhibited an enhanced suppressive capacity *in vitro*, reminiscent of data showing that polyclonal $CD4^+CD25^+$ T cells after IL-2-mediated expansion *in vitro* displayed an augmented suppressive potency (22, 23). Along the same line, it had been shown that, after transfer into IL-2 receptor $\beta^{-/-}$ mice (16) or *rag*$^{-/-}$ mice (17), polyclonal $CD4^+CD25^+$ T cells expand *in vivo* and retain their *in vitro* suppressive properties. In the latter studies, however, it was not clear whether expansion was driven by homeostatic rather than antigen-specific mechanisms.

Can our observations *in vivo* be reconciled with current hypotheses on the action of regulatory T cells based on *in vitro* data? Generally accepted hallmarks of inhibition by $CD4^+CD25^+$ T cells *in vitro* are that (*i*) these cells are anergic, (*ii*) anergy and suppression can be broken by the addition of high amounts of exogenous IL-2, and (*iii*) inhibition is contact-dependent (reviewed in refs. 3 and 8). We consider it likely that a yet-to-be-defined milieu *in vivo*, only one component of which may or may not be IL-2, would allow for the antigen-driven proliferation of $CD4^+CD25^+$ T cells. It is not clear at present whether similarly high quantities of IL-2 as used *in vitro* are available in particular microenvironments *in vivo*. The cotransfer data presented here indicate a somewhat unexpected dynamics of suppression *in vivo* in that the regulators "outgrew" the progeny of $CD25^-$ cells, which demonstrates that proliferation and suppressive function of regulatory T cells are not mutually exclusive *in vivo*.

It has been suggested that immune regulation may act at least in part through competition for growth factors and space (24, 25). $CD25^+$ regulatory T cells, which produce IL-2 only poorly or not at all, may use IL-2 and other growth factors produced by neighboring cells for their own expansion and thus deplete these factors in the local microenvironment. We found that during their expansion, $6.5^+CD4^+CD25^+$ T cells further up-regulated CD25, which should allow for a very efficient consumption of IL-2. By contrast, the presence of regulatory T cells negatively affected CD25 expression on progeny of naive T cells. This reduction in CD25 expression may indicate IL-2 "starvation,"

because IL-2 regulates its high-affinity receptor via a feedback mechanism (26). We are aware that a model that is based solely on IL-2 competition would postulate a role different from the role of IL-2 in T cell apoptosis and down-modulation of T cell responses (27–29) and could not explain the function of CD25 negative regulatory T cells (6, 30, 31).

A contribution of competition for growth factors to the suppressive action of $CD25^+$ regulatory T cells *in vivo* is not necessarily contradictory to *in vitro* findings that have been interpreted to indicate a "contact-dependent" mechanism. Thus, regulatory T cells may much more efficiently consume such factors, perhaps but not necessarily produced by the responder itself, when they are in the close vicinity of cells to be suppressed. Some quantitative considerations illustrate that the capacity of suppressor T cells to expand *in vivo* indeed may be an essential feature of immune regulation: Although the "standard" *in vitro* assay artificially generates frequencies of regulators and responders of at least 1 in 10, it seems reasonable to assume that the frequency of regulatory $CD4^+CD25^+$ T cells of a given specificity within a normal T cell repertoire is significantly lower. We consider it highly unlikely that under these circumstances a mechanism of suppression that depends on contact or close proximity can be immediately effective for instance when a self-antigen becomes exposed due to tissue damage. This scenario would explain why, under our experimental conditions (i.e., a frequency of 1 in 3000), naive cells were initially recruited into the early response irrespective of the presence of regulators of identical antigen specificity. The suppressive effect appeared to kick in once the regulatory population had reached a critical size, which may allow for a certain proximity/close contact of regulators and responders. Alternatively or in addition, "priming" of regulatory T cells may be necessary to unfold their full suppressive potential. The enhanced suppressive potency *in vitro* of expanded regulators argues in favor of such a scenario. On the side of suppressed cells, premature transition into activation-induced cell death may be a contributing factor, in particular in view of our observation that the number of the progeny of naive cells reached an early plateau in the presence of suppressors although the majority of cells continued to cycle. This finding would be consistent with the notion that some suppressed cells may die rather quickly. However, preliminary analyses did not reveal an increase in annexin V-positive cells among the progeny of naive T cells in the presence of regulatory T cells (data not shown).

Thus far, our data do not provide evidence for an "infectious" mechanism of immune regulation *in vivo* by $CD25^+$ regulatory T cells in that they may not only limit the expansion of naive cells but also may influence the effector functions (cytokine production) of suppressed T cells beyond the phase of acute suppression, as has been described recently *in vitro* for human $CD4^+CD25^+$ T cells (32). Furthermore, the role of IL-10 in the model presented here needs to be addressed further. IL-10 has been shown to be dispensable for the suppressive effect of $CD4^+CD25^+$ T cells *in vitro* (9, 10, 23) and in specific models *in vivo* (33), however, certain models of "lymphopenia-driven" autoimmunity suggest an important role for this suppressive cytokine (12, 15, 34).

Taken together, the data presented here visualize a more dynamic *in vivo* behavior of antigen-specific $CD4^+CD25^+$ regulatory T cells than previously assumed in that these cells readily expand after antigenic stimulation. It is of interest to note that Gavin *et al.* (17) recently reported contradicting observations in another TCR transgenic model after immunization with antigen emulsified in complete Freund's adjuvant. It remains open in how far factors such as prior antigenic experience, antigen dose, TCR affinity, or the number of transferred cells may account for such apparent discrepancies. With respect to the mode of antigen delivery, however, we should stress that $CD25^+$ regulatory T cells proliferated similar to naive cells irrespective of whether mice were immunized with peptide in IFA, with lipopolysaccharide-stimulated peptide-pulsed dendritic cells, or injected intravenously with peptide in PBS (our unpublished results). Based on our observations we propose that antigen-driven expansion of regulatory T cells is an essential feature of antigen-specific immune regulation because it establishes frequencies of regulatory T cells in an antigen-exposed microenvironment that may be critical for efficient suppression.

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RESEARCH ARTICLE

Gene transfer and genetic modification of embryonic stem cells by Cre- and Cre-PR-expressing MESV-based retroviral vectors

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Abstract

Background Genetic modification of embryonic stem (ES) cells represents a powerful tool for transgenic and developmental experiments. We report that retroviral constructs based on murine embryonal stem cell virus (MESV) can efficiently deliver and express Cre recombinase or a post-translationally inducible Cre-Progesterone receptor (Cre.PR) fusion in mouse fibroblasts and ES cells.

Methods To study the vectors a sensitive reporter cell line, 3TZ, was derived from the murine 3T6 fibroblast line that expresses β-galactosidase only upon Cre-mediated recombination. This was used together with the ROSA26-R ES cell Cre-reporter system or unmodified mouse ES cells as targets of infection. Efficiency of gene transfer was evaluated immunohistochemically by the use of an anti-Cre polyclonal antibody, and by monitoring the expression of β-galactosidase.

Results Infection of the 3TZ cells with high titer 718C or 719CP virus revealed efficient gene transduction of constitutive or hormone-inducible recombinase activity, respectively. The vectors efficiently transduced murine ES cells with Cre, Cre-PR (fusion of Cre and progesterone receptor) or β-galactosidase. Cre-mediated recombination in more than 60% of ROSA26-R ES cells was achieved when infected by a VSV-G-pseudotyped MESV retrovirus at MOI of 50.

Conclusions The MESV-based retroviral systems, when combined with hormone inducible Cre, represent efficient tools for the transfer of Cre activity in ES cells. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords gene transfer; ES cells; MESV retroviral vectors; adenoviral vectors; Cre recombinase; Cre.PR fusion

Introduction

Vectors based on adenoviruses or retroviruses remain the vectors of choice for gene transfer [1,2], with MoMuLV-based vectors constituting the majority of viral constructs used in gene therapy protocols up to now [3]. Such vectors are often poor in transduction of and expression in stem cells, as well as in early embryos [4–7]. In contrast to MoMuLV, the artificial murine embryonal stem cell (MESV) retrovirus can escape transcriptional blocks in undifferentiated cells [4–10]. One of the most undifferentiated experimental tools is represented by the totipotent, mouse blastocyst-derived, embryonal

stem (ES) cells [11]. Viral gene delivery to ES cells has been a great challenge because of poor infection and poor gene expression [5]. However, MESV- and HIV (lentiviral)-based vectors have been recently used for transfer of reporter protein encoding genes into ES cells with good results [12–14].

Genetic manipulation of stem cells represents an important tool in research, as well as in clinical *ex vivo* gene therapy. In combination with ES cells, the Cre/loxP system has provided a useful tool for precise genomic modifications in transgenic animals, allowing for targeted gene activation and/or inactivation strategies [15,16]. Regulation of Cre recombinase has been recently achieved by fusing Cre to modified hormone-binding domains (HBD) of steroid receptors [17–20]. In particular, progesterone, estrogen or glucocorticoid receptor HBDs containing mutations or deletions that allow the binding of synthetic hormone analogues to the HBDs, but not of the native hormones themselves, have been fused to Cre recombinase and used in numerous *in vivo* applications [21–23].

Several recent reports have documented the possibility of genetic modification of ES cells with viral vectors [8–10,12–14,24–26]. Gene transduction efficiencies have been assessed through monitoring of the expression of reporter systems or genes conferring antibiotic resistance (e.g. green fluorescent protein, neomycin phosphotransferase II gene, etc.), indicating in some instances unpredictable stability of gene expression upon differentiation of the cells [9,10,12–14]. Limited quantitative information is available on the efficiencies of gene transduction, and of comparison of retroviral versus adenoviral gene transfer. Perhaps one of the most promising aspects of viral modification of ES cells is the use of targeted recombination, which is a permanent and irreversible genetic modification.

Retroviral delivery and expression of hormone-inducible Cre in stem cells provide a powerful experimental tool. Accordingly, we have generated MESV-based vectors expressing Cre, or Cre.PR, together with a sensitive Cre-specific read-out system (3TZ murine fibroblasts), that allows the precise titration of Cre-expressing vectors. We have conducted a quantitative analysis of the ES-specific gene transfer by these vectors into mouse fibroblasts, as well as into ES cells, and, by comparing with representative MoMuLV or adenoviral vectors, we have documented the suitability of the MESV-based retroviruses in transducing and expressing in ES cells. Finally, we were able to achieve high recombination rates with these retroviruses in a functional, ES-based, Cre assay system.

Materials and methods

Cell lines

The amphotropic retroviral packaging cell line PhoenixA[31] (ΦNX-Ampho; SBR-423) was kindly provided by Dr G. Nolan (Stanford, USA). The ecotropic retroviral packaging cell line TE-FLY-E was based on the human rhabdomyosarcoma-derived TE671 cells, as previously described [27]. The cell lines mentioned above, the human embryonic kidney-derived 293 cells, the Swiss 3T6 mouse fibroblast cell line, as well as their derived clones, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). ROSA26-R ES cells (a kind gift from Dr P. Soriano [28]) and the HM-1 mouse embryonic stem cell line [29], kindly provided by Dr T. Magin, were maintained on mouse embryonic fibroblasts as a feeder layer, in BHK-21 medium, supplemented with 15% (v/v) FCS, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 0.1 mM NEAA, as well as 1000 U/ml leukemia inhibitory factor (LIF, Gibco) to maintain the undifferentiated phenotype. Feeder cells were prepared from 12–14-day-old C57Bl6 embryos. Growth arrest was achieved by culturing the cells in the presence of 10 μg/ml mitomycin C for 3 h.

Retro- and adenoviruses

Plasmids

All retroviral vectors constructed were based on the chimeric vector p50M [7,9]. For the construction of the p718C vector, a 1.1-kb XbaI(blunted)-EcoRI fragment containing the Cre recombinase gene fused with a nuclear localization signal [30] (NLS-Cre) was cloned into the XhoI(blunted)-EcoRI sites of the parental vector. p719CP vector was constructed by insertion of a 1.9-kb XhoI-Bgl II fragment from plasmid pBSMcCrePR [18], containing the NLS-Cre.PR sequence, in the XhoI-BamHI sites of the parental vector. pBSMcCrePR contains an inducible fusion between the Cre gene and a mutated form (hPR891) of the hormone-binding domain (HBD; aa 641–891) of the human progesterone receptor which lacks 42 amino acids from its C-terminus. This deletion renders PR unable to bind and respond to progesterone, yet it is still able to bind and respond to the synthetic steroid RU486 [18]. p796L vector was constructed by inserting a 3.3-kb BamHI fragment containing the nls-lacZ sequences in the BamHI site of the parental vector.

The BAG vector expresses β-galactosidase in the MoMuLV context [31]. H5.010CMVlacZ, an E1-deleted Ad5 adenovirus expressing nuclear lacZ under the CMV promoter, was kindly provided by Dr Karin Jooss [32].

Packaging

For the packaging of the retroviruses we used the ecotropic TE-FLY-E packaging cell line, the amphotropic Phoenix A packaging cell line and the VSV-G pseudotyping system [27,33,34].

For the generation of ecotropic Cre-expressing viruses, TE-FLY-E cells were plated in 60-mm dishes at 7×10^3 cells/cm^2 and transfected by the calcium phosphate method [35] using 7 μg of the viral vector plasmid together with 1 μg of the pSV2-neo plasmid (Stratagene)

carrying the neomycin phosphotransferase II gene. Neomycin-resistant colonies were selected against G418 (800 μg/ml) and further expanded.

Positive clones were further checked for Cre expression by hybridization of viral RNA, isolated from supernatants of the clones, with a Cre-specific probe. Briefly, 2 ml medium without G418, conditioned for 48 h with subconfluent cultures of each individual clone grown in 25-cm^2 flasks, were collected, filtered through a 45-μm filter, incubated for 30 min at 37 °C in 10 mM EDTA, 100 μg/ml proteinase K, 0.5% (w/v) SDS, acid phenol and chloroform extracted and ethanol precipitated together with 5 μg tRNA as carrier. RNA was resuspended in 50 μl TE buffer, 75 μl formamide and 25 μl formaldehyde (37%, v/v) were added, and samples were incubated for 10 min at 60 °C. The RNA was then diluted in 10× SSC, applied in 1:3 serial dilutions to a dot blot apparatus on nitrocellulose membrane and hybridized with a Cre-specific probe according to Church and Gilbert [36].

The viral supernatants were in parallel subjected to titration of their infectious activity using the especially developed Cre-activity read-out system of 3TZ fibroblasts. Cre producer clones revealed by both methods are further referred to as TEC cells (for TE-FLY-E generated, MESV-based, Cre-expressing retrovirus). Best clones (e.g. TEC3) were further expanded and viral supernatants were collected from confluent cultures, titrated and used throughout this study.

Alternatively, TE-FLY-E cells were plated at 100, 10 or 1 cells/well in 48-well plates and infected with amphotropic virus containing supernatant produced by transient transfection of PhoenixA with p796L vector plasmid as described below. The titers of the emerging clones were estimated using 3T6 cells as read-out system and lacZ producers are further referred to as TEZ clones (for TE-FLY-E generated, MESV-based, LacZ-expressing retrovirus), unless specified otherwise.

The amphotropic viruses were produced by transiently transfecting 10 μg of each plasmid into Phoenix A cells plated in 60-mm dishes at 7×10^4 cells/cm^2 by the chloroquine/calcium phosphate method, as described [37]. Fresh medium (3 ml) was added 1 day post-transfection and collected 48 h later. Supernatants were filtered through a 45-μm filter and titers were estimated using the 3TZ read-out system (see below).

For the VSV-G pseudotyping, 293 cells were transiently transfected as described above with the pMD.gagpol and the pMDtet.G plasmids (expressing the MoMuLV gag-pol and the VSV-G protein, respectively, both kind gifts of Dr R. Mulligan [34]) together with the retroviral vector. Supernatants were collected 48 h later and were further handled as described [38].

Viral infection

3T6 or 3TZ cells were plated at 1.0×10^4 cells/cm^2; HM1 ES cells were plated at $0.35–1.0 \times 10^5$ cells/cm^2 on feeder cells (1.0×10^5 feeder cells/cm^2) in 6- or 24-well plates. Cells were infected the next day in the presence of 8 μg/ml polybrene with serial dilutions of the respective viral supernatants, representing thus different multiplicity of infection (MOI) ratios, calculated against cell numbers at the time of infection. Then, 12–16 h later, medium was changed and Cre or β-galactosidase expression was detected 2 days later by either X-Gal staining or staining with an anti-Cre antibody (see below). Viral titers (IU/ml; infectious units contained per ml of supernatant) were calculated by multiplying the numbers of transduced cells by the dilution factor of the supernatant. For the 719CP virus the term 'Cre-active particles' was used, taking into account that the numbers of cells expressing Cre activity in the absence of RU486 do not reflect the actual transduction efficiency seen in the presence of the hormone analogue, but rather the fraction of cells transduced with a leaky transgene. For adenoviral infections an identical infection protocol was followed, including the presence of polybrene, which has been shown to facilitate adenovirus-mediated gene transfer in various cell lines [39].

Transfection

3TZ cells were plated at 1.0×10^4 cells/cm^2 in 6-well plates. The next day, the cells were transfected with 5 μg of p718C or p719CP plasmid DNA using the calcium phosphate method [35]. Twenty-four hours later, cells were washed with PBS, incubated for 10 min at 37 °C with HBSS solution (Gibco) and further incubated with normal growth medium with or without 0.1 μM RU486 (Sigma, St. Louis, WI, USA). Forty-eight hours later, cells were fixed and stained with X-Gal for detection of β-galactosidase activity (see below).

Generation of a Cre activity reporter system

For the generation of the 3TZ Cre activity read-out system, 10^7 mouse 3T6 fibroblasts were suspended in 0.8 ml PBS and electroporated (BioRad gene pulser; 960 μF, 300 V) with 4 μg of the NotI-linearized pψβ-galoxé plasmid, which expresses β-galactosidase only after Cre-mediated recombination [18] (see Figure 1B). Stable clones were selected against G418 (800 μg/ml) and further expanded. Positive clones were plated in duplicates in 24-well plates at 2×10^4 cells/well and infected with Cre-expressing retroviral particles at MOI of 10 or left untreated. Forty-eight hours after infection, cells were fixed and stained with X-Gal. Only clones with the absence of any β-galactosidase activity in the untreated cultures and 100% blue-stained cells in the infected ones were further processed for Southern analysis (EcoRI digestion) performed by standard procedures.

Detection of Cre expression with anti-Cre antibody

Cells were plated on coverslips at 60–80% confluency. On the next day they were washed twice with PBS, fixed

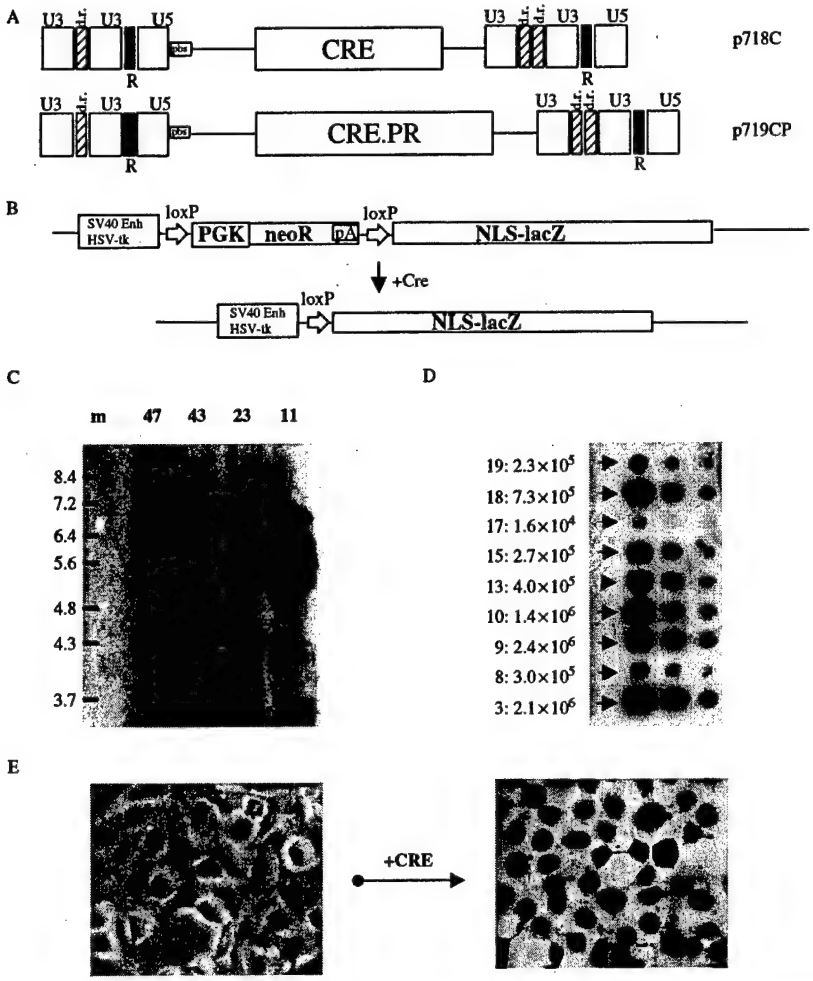

Figure 1. Generation of retroviral vectors expressing Cre recombinase. (A) Chimeric retroviral vectors expressing constitutive (p718C) or RU486-inducible (p719CP) Cre recombinase; pbs: primer binding site (here: MESV pbs for $tRNA^{glu}$); d.r.: direct repeat; U3, R, U5: regulatory elements of MoMuLV-based LTRs. The 5′ LTR, containing one characteristic direct repeat sequence in the U3 region, is from PCMV whereas the 3′ LTR, containing two direct repeats, is from MPSV. The vectors are depicted before integration. (B) Schematic representation of the construct used for the generation of the Cre-specific read-out system: Nuclear lacZ is expressed under HSV-tk promoter and SV40 enhancer elements only after a Cre-mediated loxP recombination event, which leads to the excision of the neoR unit. neoR: neomycin phosphotransferase II gene; PGK: phosphoglycerate kinase promoter; pA: SV40 polyadenylation signal. (C) Southern analysis of 3TZ clones: 3T6 cells were stably transfected with the Cre-reporter construct of (B). DNA from individual, G418-resistant clones (3TZ cells) were digested with EcoRI, and further subjected to Southern analysis using a lacZ-specific probe, to test for the integrity of the reporter construct. m: molecular weight marker in kb. (D) Packaging of the Cre retrovirus: dot blot of viral RNA (1 : 3 serial dilutions) isolated from the supernatants of nine individual clones (3, 8, 9, 10, 13, 15, 17, 18 and 19) of TE-FLY-E packaging cells stably transfected with p718C. RNA was immobilized on nitrocellulose membrane and hybridized to a ^{32}P-labelled Cre-specific probe. The titers in IU/ml were estimated using the Cre-specific 3TZ read-out system. (E) Cre-reporter functional assay: non-treated (left) or virus-infected (right) 3TZ 43 cells were fixed, stained overnight with X-Gal (1 mg/ml) staining solution and photographed. 100% of the cells of the clone 3TZ 43 express β-galactosidase in the nucleus after infection with Cre retrovirus at MOI 10, whereas the non-treated ones show absolutely no background. Original magnification ×50

for 10–15 min at room temperature (RT) with 3.7% (v/v) formaldehyde in PBS, washed three times with PBS, incubated for 5 min at RT with blocking buffer (250 mM KCl, 40 mM Hepes pH 7.9, 0.5% (w/v) BSA, 0.5% (v/v) gold fish gelatin, 0.1% (v/v) Triton X-100), incubated for 45 min at RT with 1 : 2000 dilution of a rabbit anti-Cre polyclonal antibody (PRB-106C; Covance, Princeton, NJ, USA) in blocking buffer, washed three times in blocking buffer, incubated for 45 min at RT with 1 : 100 dilution of phycoerythrin-labelled goat anti-rabbit IgG (GAR-PE, stock 1 mg/ml; Molecular Probes) in blocking buffer, washed three times in blocking buffer, once in PBS, incubated for 5 min with 4,6-diamidino-2-phenylindole (DAPI) in PBS, washed three times in PBS and placed

on microscope slides with a drop of mounting medium (Immuno Fluore; ICN). Fluorescence was detected using a Leitz DMRB fluorescence microscope.

Detection of β-galactosidase-induced expression with X-Gal staining

Cultures were washed with PBS, fixed for 10 min with 3.5% (v/v) formaldehyde, 0.5% (v/v) glutaraldehyde, washed twice with PBS and stained from 4 h to overnight in X-Gal staining solution (2 mM $MgCl_2$; 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$; 0.01% (w/v) sodium deoxycholate; 0.02% (v/v) Nonidet P-40; 1 mg/ml X-Gal). Cells expressing β-galactosidase were counted under a Leica DM IL light microscope.

Results

MESV-based retroviral vectors for Cre delivery and 3TZ read-out system for Cre activity

For the present study we constructed retroviral vectors based on the p50M chimeric vector of the MESV type, which, in contrast to its prototype MoMuLV, has been reported to express in undifferentiated cells [5,7–9]. Expression of this vector in ES cells benefits from the use of LTR sequences from MoMuLV derivatives, namely the myeloproliferative sarcoma virus (MPSV; at the 3′ LTR) and the PCC4-cell-passaged MPSV (PCMV; at the 5′ LTR, Figure 1A), as well as from the replacement of its natural tRNA binding site (pbs) by the one of the endogenous murine dl-587rev retrovirus [7–9].

Two types of vectors were generated using the p50M backbone (Figure 1A): (a) p718C: encoding the Cre recombinase gene, and (b) p719CP: encoding an inducible fusion between the Cre gene and a mutated form of the hormone-binding domain (HBD; aa 641–891) of the human progesterone receptor (Cre.PR). In the absence of ligand, Cre is inactive within the Cre.PR, due to the interaction of the HBD moiety with the steroid receptors regulatory system, whereas, upon RU486 binding, it passes to the active state.

Viral particles were generated from stable co-transfection of TE-FLY-E ecotropic packaging cells with vector and a plasmid conferring neomycin resistance, as well as transient transfection of the PhoenixA amphotropic packaging cell line. 12 out of 20 p718C transfected, neomycin-resistant clones produced retroviral particles containing the Cre gene (Figure 1D and data not shown). Comparable viral titers were obtained by transfecting the p719CP or p718C construct in the PhoenixA amphotropic packaging cell line (4.1×10^5 IU/ml; Figure 2B).

To titrate the supernatants for delivery of Cre activity, the 3T6 murine fibroblast cell line was transfected with a Cre-dependent β-galactosidase expression cassette (3TZ

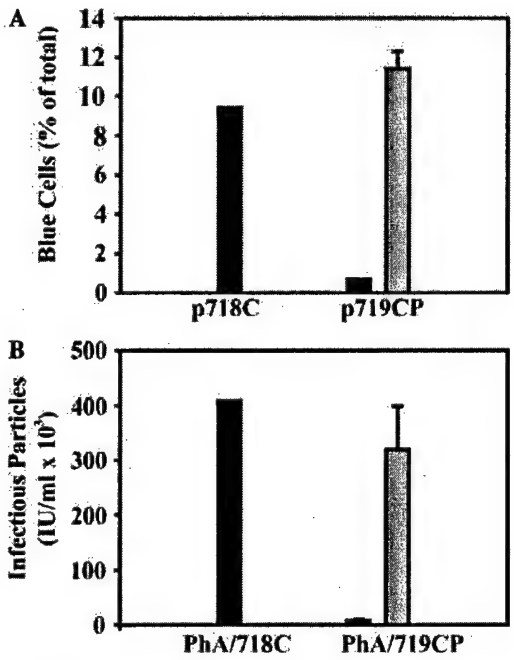

Figure 2. Inducibility of Cre.PR in the retroviral context. (A) Transfection. 3TZ cells were transfected with either p718C or p719CP using the calcium phosphate method. Cultures were further grown in the presence or absence of 0.1 μM RU486. Forty-eight hours later the percentages of total cell population which expressed β-galactosidase were calculated. Mean of two representative experiments. Grey bars: 0.1 μM RU486. Black bars: no hormone added. (B) Infection. 3TZ cells were infected in the presence of 8 μg/ml polybrene with supernatants from PhoenixA cells previously transfected with either p718C or p719CP. Cultures were grown for 48 h in the presence or absence of 0.1 μM RU486. Apparent titers in IU/ml were estimated as described in Materials and methods. Mean of three representative experiments. Grey bars: 0.1 μM RU486. Black bars: no hormone added

cells). In the Cre-dependent, β-galactosidase expression cassette, nls-lacZ is separated from an active promoter by a neomycin phosphotransferase II (neo^R) minigene (Figure 1B). Upon Cre-mediated recombination, the loxP-flanked neo^R minigene is excised and lacZ is expressed from the HSV-tk promoter and SV40 enhancer elements.

Clone 43 (3TZ43) contained only intact copy/copies of the transfected DNA (Figure 1C), which remained stable throughout extensive *in vitro* serial passaging, and showed no background of β-galactosidase in the absence of Cre. On the other hand, 100% of these cells were able to express β-galactosidase upon expression of Cre (Figure 1E and data not shown), thus avoiding contaminating, non-staining cell populations observed in similar read-outs [43], possibly resulting from tandem arrays of the integrated reporter construct [44]. The uniform nuclear β-galactosidase staining of the 3TZ 43 cells provides a highly sensitive *in vitro* assay for the detection of Cre activity expressed by both amphotropic and ecotropic retroviruses, an ability not shared by other

Cre-reporter systems based on simian [18,44,45], feline [46] or human cell lines [47].

Validation of MESV vectors and delivery of Cre activity in 3TZ cells

Using the 3TZ read-out system, the titers of supernatants, produced by several TE-FLY-E clones stably transfected with p718C (TEC cells), were estimated to range from 1.6×10^4 to 2.4×10^6 IU/ml. These results were in agreement with the intensities of the respective RNA dot blots, resulting from viral RNA hybridized with a ^{32}P-labelled Cre-specific probe (Figure 1D and data not shown). When cultured at higher cell densities the high producer TEC3 clone was able to generate viral titers up to 2.6×10^7 IU/ml, this being up to an order of magnitude higher than the titers reported for earlier vectors expressing Cre together with selectable markers [48].

Transfection and viral gene transduction were compared for delivering tight hormone-dependent regulation of Cre activity with the p719CP construct. Transient transfection of the 3TZ 43 cells line with p718C or p719CP DNA in the presence of 0.1 μM RU486 revealed comparable levels of Cre activities, with 9.4 and 11.5% of the cells staining for β-galactosidase activity, respectively. Detectable levels of hormone-independent Cre activity were observed in p719CP-transfected cells, with 0.8% of the cells expressing β-galactosidase in the absence of RU486 (Figure 2A). This corresponds to 8.5% of the total transfected cells, as estimated from the number of blue-stained cells produced by transfecting 3TZ cells with the p718C. Leaky Cre.PR activity was reduced to 2.4% of total transfected cells upon viral gene transduction. This allowed for a more pronounced relative induction of Cre activity in the presence of RU486 in virally transduced cells (average: 40.8-fold), as compared with transfected cells (average: 15.5-fold). These results demonstrate tight RU486-dependent regulation of Cre.PR activity in retrovirally transduced cells.

Comparison with Moloney retroviral and adenoviral vectors expressing constitutively active LacZ in ES cells

In order to directly quantitate the MESV-mediated gene transduction into ES cells, the p796L retrovirus encoding *E. coli* LacZ was constructed using the same retroviral vector backbone as the p718C virus (Figure 4A). Infectious particles were generated using the TE-FLY-E packaging cell line, and stable clones (TEZ) were isolated that produced viral titers of up to 0.9×10^7 IU/ml. To compare efficiencies of amphotropic and ecotropic env in mediating infection of ES cells, the p796L virus was also packaged transiently using the PhoenixA cell line (PhA/796L) and equal numbers of viral particles as assayed on 3T6 cells were used to infect HM1 ES cells. Infection of HM1 ES cells by TEZ/796L or PhA/796L retrovirus particles revealed comparable gene transduction efficiencies of 5.3 ± 0.4 and 5.05 ± 0.15%, as compared with infection of 3T6 cells, respectively (Figure 4C), indicating that both ecotropic and amphotropic pseudotyping are suitable for this purpose.

The relative transduction efficiency was unchanged (5.55 ± 0.85%) when 20 times more TEZ/796L packaged viral particles were used (Figure 4B). In contrast, infections by the BAG retroviral vector (MoMuLV-based retrovirus encoding lacZ) [31] packaged in PhoenixA cells (PhA/BAG virus) resulted in the transduction of only 0.06 ± 0.035% of the ES cells, as compared to fibroblasts. These results are in agreement with MESV being significantly more efficient than MoMLV in transducing ES cells.

We next compared the 796L retrovirus to an adenoviral vector expressing β-galactosidase under the cytomegalovirus (CMV) immediate early promoter (H5.010CMVlacZ [32], referred to here as AdlacZ). By conducting a series of infections at a variety of MOIs (ranging from 1:17 to 14:1) we observed that both viruses were able to transduce β-galactosidase activity into HM1 ES cells, with the absolute numbers of β-galactosidase-expressing ES cells ('blue' cells) increasing as the quantities of added fibroblast active viral particles increased (data not shown), this being at variance with a previous work reporting the failure of a similar AdlacZ virus to transduce undifferentiated ES cells [26]. However, AdlacZ transduced preferentially the mouse embryonic fibroblasts (MEFs) used as feeders for the HM1 ES cells (Figures 4E and 4G). By averaging the results of several experiments conducted at different MOIs (ranging from 1:7–14:1), it was estimated that 53.15% (±4.35) of the total X-Gal-stained cells per culture, after AdlacZ treatment, were morphologically MEF feeder cells (Figures 4D and 4F). This result is even more striking when considering that the MEFs represented only 11–16% of the total mixed ES and MEF cell population.

MESV-based vectors can efficiently deliver Cre to ES cells, and promote Cre-mediated recombination in ROSA ES cells

The ability of p718C to transduce Cre recombinase into ES cells was tested using a polyclonal antibody against Cre. This antibody was able to detect Cre recombinase in TE-FLY-E cells stably transfected with p718C (TEC clones). In particular, immunohistochemical staining of the high-producer TEC3 clone revealed the presence of nuclear localized Cre in 100% of the cells, whereas untransfected TE-FLY-E cells did not stain (Figure 3B and data not shown). Similarly, HM1 ES cells previously infected with TEC3 supernatants were specifically stained by the Cre antibody, indicating that the MESV-based p718C retroviral vector efficiently transfers Cre recombinase in ES cells (Figure 3D and data not shown).

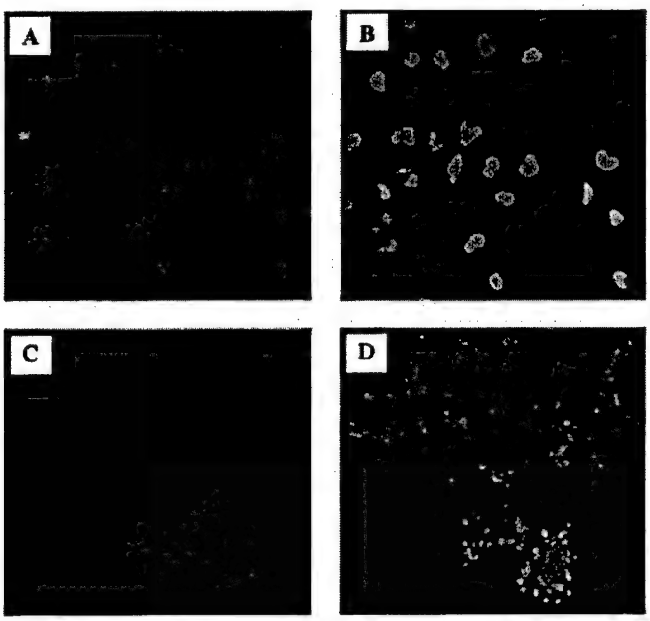

Figure 3.

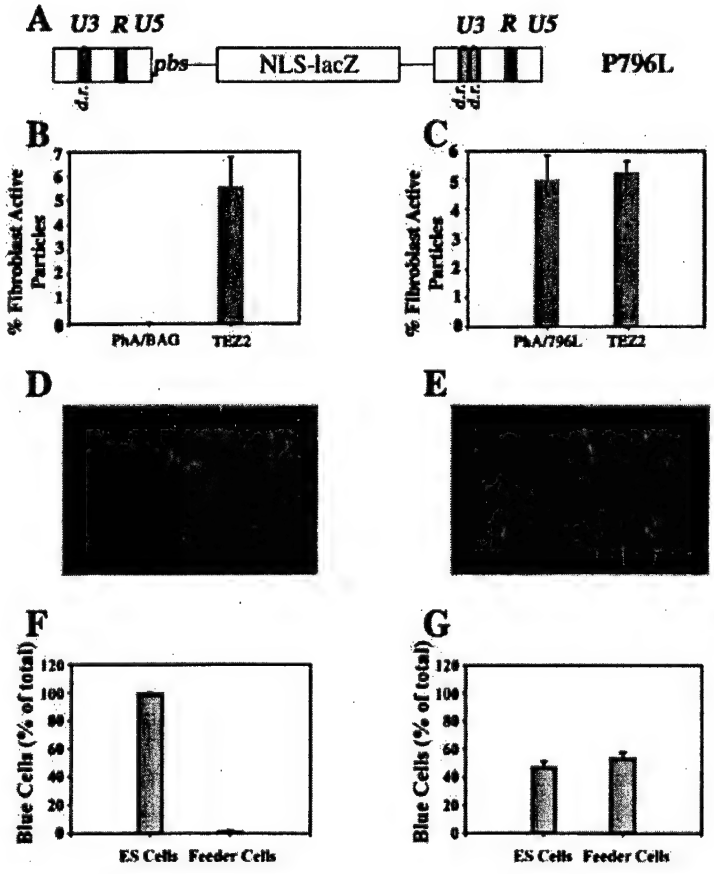

Figure 4.

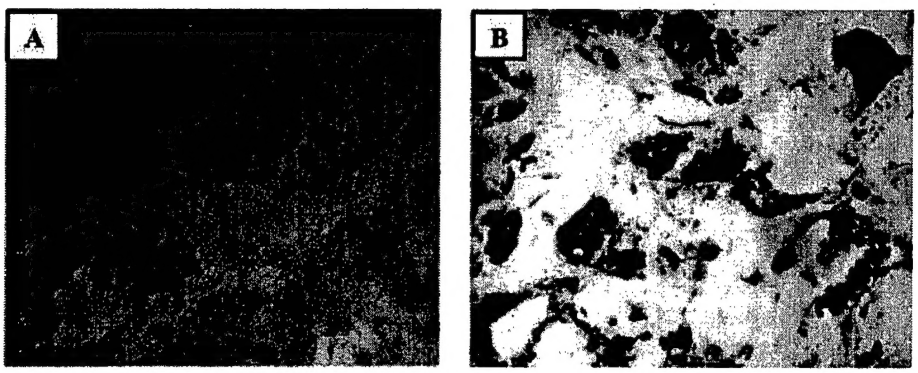

Figure 5. High efficiency MESV-mediated transfer of Cre recombinase to ES cells. ROSA26-R ES cells were plated at 10^4 cells/cm^2 in 6-well plates on feeder fibroblasts. One day later they were infected with VSV-G-pseudotyped Cre retrovirus at MOI 0.5 (A) or 50 (B) in the presence of 8 μg/ml polybrene. Two days later cells were fixed, stained overnight with X-Gal (1 mg/ml) staining solution and photographed. Original magnification ×100

To test the ability of this retroviral system to transfer Cre activity in ES cells, we used the ROSA26-R ES cell read-out system, and VSV-G pseudotyped virus, which allows pelleting of the virus by ultracentrifugation and virus concentration without loss of activity [38]. Up to 20 times higher titers were achieved by this method, reaching 5×10^8 3TZ infectious units per ml. Using high titer virus, at MOI 50, functional recombination was achieved in the majority of ES cells (Figure 5B). At MOI 0.5, 1.54 ± 0.71% of the population, and, at MOI 0.005, 0.21 ± 0.02% of ES cells were transduced, suggesting that transduction of ES cells by the VSV-G-pseudotyped virus is disproportionate to the input of Cre-expressing viral particles, as was also the case for the MoMuLV-enveloped virus (not shown).

Discussion

The goal of the present work was to generate and analyze viral vectors that efficiently express Cre and hormone-inducible Cre in embryonic stem cells. Viral titers were compared using a novel Cre-reporter system based on 3T6 fibroblasts, which we showed are also susceptible to infection with adenoviral vectors. Similar read-out systems derived from NIH-3T3 [40] have been reported, which are likely to lack adenovirus receptors [41,42].

Our finding that retroviral transduction of mouse fibroblasts with p719CP allows for tighter regulation of the expressing Cre.PR by RU486, as compared with transfection techniques, is of importance for the transfer of this and other post-translationally controlled Cre systems in cells. The higher leakiness observed upon transfection of the vector DNA may be due to several reasons: (1) The stress of transfection may transiently interfere with the mechanism of regulation of the steroid receptors. (2) Large quantities of DNA taken up by the cells may lead to high levels of expression of Cre.PR, thus overwhelming the natural regulation of steroid

Figure 3. Detection of Cre recombinase in ES cells infected with TEC3 supernatants. (A, B) TEC3 cells were plated on glass coverslips at 1.0×10^4 cells/cm^2. Next day they were immunostained with a rabbit anti-Cre polyclonal antibody. Nuclei stained with DAPI were identified under UV light (A), whereas nuclear localized Cre could be visualized after staining with a phycoerythrin-labelled secondary antibody (B). Original magnification ×40. (C, D) HM1 ES cells were plated on glass coverslips at 0.5×10^5 cells/cm^2. The next day they were infected with TEC3 supernatants in the presence of 8 μg/ml polybrene. Two days later cells expressing Cre were immunostained with a rabbit anti-Cre polyclonal antibody followed by a phycoerythrin-labelled secondary antibody (D). Nuclei were visualized using DAPI staining (C). Original magnification ×20

Figure 4. Differential pattern of infection of HM1 ES cells by 796L and AdlacZ viruses. (A) MESV-based retroviral vector expressing *E. coli* β-galactosidase containing a nuclear location signal (p796L); pbs: primer binding site (here: MESV pbs for tRNA^{glu}); d.r.: direct repeat; U3, R, U5: regulatory elements of MoMuLV-based LTRs. (B) HM1 ES cells were plated on feeder cells. The next day they were infected in the presence of 8 μg/ml polybrene with 1×10^4 'fibroblast-active particles' of either PhA/BAG or TEZ2/p796L virus. Two days later cells were fixed and stained with X-Gal staining solution. Numbers of blue cells were expressed as percentages of the input fibroblast-active particles. Mean of three independent experiments. (C) HM1 ES cells were plated on feeder cells. The next day they were infected in the presence of 8 μg/ml polybrene with 2.5×10^5 'fibroblast-active' particles of either PhA/796L or TEZ2/p796L virus. Two days later cells were fixed and stained with X-Gal staining solution. Numbers of blue cells were expressed as percentages of the input fibroblast-active particles. Mean of two independent experiments. (D, E) HM1 ES cells were plated at 0.35×10^5 cells/cm^2 on feeder cells (previously plated at 1.0×10^5 cells/cm^2). The next day they were infected in the presence of 8 μg/ml polybrene with 5×10^5 'fibroblast-active' particles of either TEZ2 (D) or AdlacZ virus (E). Two days later cells were fixed and stained with X-Gal staining solution. Original magnifications ×50. (F, G) Percentages of total X-Gal-stained cells identified as blue-stained ES cells and blue-stained feeder cells, respectively. Mean of two representative retroviral TEZ2 infections (F) and eight adenoviral AdlacZ infections (G)

receptors [53]. (3) Mutations, rearrangements or partial degradation of DNA taken up by the transfected cells may inactivate or delete the PR domain in a fraction of the expressed proviral DNA. In contrast to DNA transfection, retroviral gene transduction characteristically leads to the integration of only a limited number of intact functional copies, with minimal stress for the target cells.

Retroviral vectors transduce ES cells considerably less efficiently than fibroblasts [10,12]. Similar results were obtained even when transgene expression was driven from internal promoters theoretically avoiding the LTR-mediated transcriptional silencing in ES cells [25]. On the other hand, Laker *et al.* [9], measuring neo resistance conferred from monocistronic MESV vectors, reported comparable efficiencies of gene transduction for either ES cells or NIH3T3 fibroblasts, whereas bicistronic vectors preferentially transduced the fibroblasts. Variations in the relative efficiencies of transduction into ES cells could in part be attributed to the different titration methods used, to differences in construct characteristics such as splicing signals and number of cistrons included, or to the different ES cell lines used [8].

The poor gene expression afforded by MoMuLV-derived vectors in embryonic carcinoma (EC) and ES cells [5] is partially circumvented by MESV-based [8–10] or MND [10] vectors. We were able to show that the MESV-based retrovirus can transfer Cre activity into ES cells. This was shown both by immunostaining documenting the presence of Cre protein in the target cells, as well as by assessing its recombinogenic activity in a functional ES cell assay. The latter system allowed us to achieve high (over 60%) transduction efficiencies.

By infecting with equal quantities of retroviral particles, estimated from transduction of mouse fibroblasts, we were able to calculate that MESV-based vectors can transduce the ES cells approximately by two orders of magnitude more efficiently than vectors containing original MoMuLV LTRs and pbs. This improvement is substantially stronger than that observed by Robbins *et al.* [10] who, using a MoMuLV-based vector expressing the chloramphenicol acetyltransferase (CAT) gene, estimated a 12% relative CAT activity in CCE ES cells compared with PA317 fibroblasts and only a 4-fold improvement with the use of the MESV-like, MND vector. Others have reported failure of MoMLV-driven transgene expression in ES cells [12].

Our findings are consistent with results recently reported for transduction of fluorescent markers into ES cells using retroviral vectors [12–14]. It should be noted, however, that efficiencies reported here have been achieved in some cases only when exposing the cells to extremely high excess of viral particles (e.g. at MOI nearly 600 [13]), whereas in other systems more modest conditions are required [14]. Insertion of regulatory elements such as the woodchuck hepatitis B virus post-transcriptional regulatory element or VSV-G pseudotyping was reported to increase efficiency of transduction to levels comparable to those mediated by lentiviral vectors [13,14].

Comparison of 796L with AdlacZ-mediated gene transfer into ES cells revealed that the retroviral vector preferentially transduced ES cells, as compared with the AdlacZ vector that transduced preferentially the MEFs used as feeders in the ES culture. The preferential infection of MEFs could potentially be related to the fact that the MEFs were mitotically blocked by mitomycin treatment [49] as well as to a relative abundance of the main adenovirus receptor, the CAR receptor [42], in MEFs as compared to ES cells. Variable expression of this receptor has been recently shown for other cell lines [50,51]. By analogy, the relative failure of adenoviral vectors to transduce another type of stem cell, the hematopoietic stem cell, has been attributed to low or undetectable adenovirus receptor levels [52]. It should also be noted that our relative efficiency of ES transduction by AdlacZ (as compared with the transduction of fibroblasts infected by the same vector) was significantly lower than the 30–50% efficiency reported for the infection of AB1 ES cells at the high MOI of 100, with an adenoviral vector expressing neo^R under the PGK promoter [24]. One reason for this may be more efficient activity of the PGK promoter as compared with that of the CMV promoter in ES cells. However, the estimations in that report were deduced from Southern blot analysis, essentially differing from our assay. Nevertheless, the CMV promoter is the most commonly used internal promoter in adenoviral vectors in numerous *in vivo* applications [3].

Transgene expression from retrovirally modified ES cells is silenced in the emerging mice through both methylation-dependent and -independent mechanisms, even when the retrovirus used is of the MESV-type [12]. Accordingly, this transcriptional repression can be partially circumvented by the use of 5-azadeoxycytidine. MESV-driven transgene expression was reported to be maintained upon differentiation of ES cells or hematopoietic progenitor cells [12], allowing the use of retrovirally modified stem cells for developmental experiments, as a viable alternative to the recently established lentivirus-based systems [13]. Transfer and activation of Cre in appropriately modified stem cells allow for irreversible site-directed mutagenesis, which in any case does not suffer from shut down of retroviral gene expression afterwards. The post-translational regulation of Cre-mediated recombination in ES cells also limits the timing, extent and the potential toxic effects of Cre activity [54]. The fact that Cre.PR can be efficiently activated in the presence of RU486 in murine ES cells [18] renders this regulatory system a powerful tool for inducible site-specific recombination in embryonic and other stem cells.

In summary, using the Cre- or Cre.PR-expressing p718C and p719CP retroviruses we were able to achieve efficient gene transduction and high Cre/loxP recombination rates in two different ES cell lines. These studies indicate that the MESV-based retroviral vectors are suitable vectors for gene transfer and genetic modification of ES cells.

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